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Mitotic Triggers in the Cell Cycle: Phase Dependency of Cleavage Advance on the Chromosome Cycle in Parthenogenetically Activated Sea Urchin Eggs.

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MITOTIC TRIGGERS IN THE CELL CYCLE: PHASE DEPENDENCY OF CLEAVAGE ADVANCE ON THE CHROMOSOME CYCLE IN PARTHENOGENETICALLY ACTIVATED SEA URCHIN EGGS

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ABSTRACT

In the sea urchin egg, metabolic activation, DNA synthesis and the chromosome cycle can be induced with parthenogenic activators (NH_4Cl or procaine) although no spontaneous cleavage will occur. To determine the effects of these agents on other cell cycle events, eggs from four species of sea urchins were activated with NH_4Cl or procaine and then fertilized at different times of the chromosome cycle. These eggs show a decrease in the time to first cleavage which is apparently coupled to the parthenogenically induced chromosome cycle. The advance is seen whether the activated eggs are fertilized during the first or second chromosome cycle. This chromosome cycle consisting of distinct condensation and decondensation phases can be uncoupled by selective removal of the agent. These removal effects are time dependent; once a cell has reached a critical point the chromosomes are committed to decondense. However, earlier removal results in maximally condensed chromosomes which do not decondense. In either case, if these eggs are then fertilized, cleavage advance is not affected, demonstrating

that it is independent of chromosome decondensation.

To determine whether cleavage advance is directly linked to other cycle related events, eggs were activated while in the presence of protein synthesis inhibitors cycloheximide and emetine. Results using these inhibitors indicates that chromosome condensation, but not decondensation, can occur in activated, inhibited eggs. There is no cleavage advance in these eggs. Activation of eggs by either NH_4Cl or Ionophore A23187 in Na and/or Ca free sea waters with protein synthesis inhibitors suggests that initiation of condensation is dependent upon cytoplasmic pH, but not upon external Na or Ca ions.

Studies of the effects of NH_4Cl on surface morphology were also undertaken. NH_4Cl induces microvillar elongation approximately 60 minutes after addition. Longitudinally oriented microfilaments are observed in Transmission electron micrographs of elongated microvilli. If NH_4Cl is removed, microvilli shorten in length and increase in diameter. No microfilaments are visible in short microvilli. When NH_4Cl is subsequently readded, microvilli elongate, becoming highly branched and/or knobbed in appearance. Microfilaments are observed in these elongate microvilli.

Chapter 1

OVERVIEW

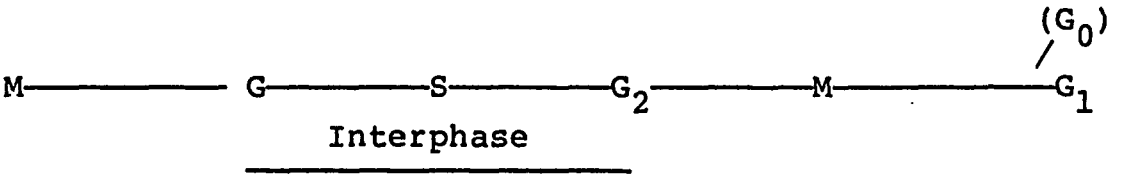
PREFATORY COMMENTS

The intrinsic factors controlling cellular cycles have long evaded understanding. The complexity of spatial and temporal interactions among cytoplasmic, nuclear, organellar and surface components which govern the life of a cell, and culminate in it's division, contraindicates a simple, pervasive solution. Integration of the large body of literature now available with regard to metabolic and biochemical events involved in cell cycle regulation will be difficult. Even then the complexities involved suggest any single functional switch activating the cell to mitosis will be difficult to define.

1. The Cell Cycle

The cell cycle is the life of an individual cell, and represents all of the events occurring in sequence from one cellular division to the next. It is a repetitive series of events, hence the term cell cycle. The basic eucaryotic cell cycle is generally subdivided into four distinct phases referred to as

G_1, S, G_2 and M, which are schematically represented as follows:



Interphase (encompassing G_1, S and G_2) is actually a dynamic state of the growing, metabolically active cell. The G_1 phase (or Gap 1) marks the interval occurring between the end of mitosis and the onset of the synthetic phase (S). It is during this portion of the cycle that synthesis and mobilization of the various elements required for DNA synthesis occurs (Wilson, 1966). S-phase represents the portion of the cycle during which DNA replication takes place (Baserga, 1969), while G_2 (Gap 2) is a relatively nonvariable interval occurring from S to mitotic onset (Wilson, 1966; Mitchison, 1971; Baserga, 1969; Mazia, 1974).

Mitosis (M) consists of those typical stages ensuing as the cell progresses in an orderly manner from prophase through telophase. The chromosomal configurations and movements involved in M have been described in detail, and are well summarized by Wilson (1966) as consisting of:

1. Prophase - the unwinding and contracting of chromosomes.

2. Prometaphase - nuclear boundary breakdown, collapse of chromosomes to a central clump, disappearance of the nucleolus.

3. Metaphase - positioning of chromosomes along the equatorial spindle plate.

4. Anaphase - separation of chromatids.

5. Telophase - reformation and restoration of nuclear integrity and the nucleoli.

Also associated with these stages on a temporal basis are protein synthesis, beginning in telophase and continuing to early prophase, and RNA synthesis, occurring in late telophase or early interphase (see Wilson, 1966; Baserga, 1969; Mitchison, 1971 for reviews)

In the sea urchin egg, the first cell cycle is represented by the events initiated at sperm-egg contact resulting in metabolic activation of the quiescent egg, and culminating in first division. Studies involving these eggs as model systems are complicated by the gross number of events initiated at fertilization which cannot in reality be separated from the basic mitotic events (Harris, 1969) and by the fact that cell cycles of sea urchin eggs are somewhat atypical relative to most higher eukaryotic cycles (Mazia, 1974; Mitchison, 1971). In urchin eggs, the chromosome cycle is fairly rapid (Mazia, 1974) and DNA synthesis occurs only during a short interval, beginning a few minutes after telophase

(Hinegardner et al., 1964). S-phase is succeeded by a G_2 of approximately 20 minutes duration (in Strongylocentrotus purpuratus at 15° C). Subsequent to G_2, mitosis of approximately 40 minutes duration occurs. There is little or no G_1 in cycles following the first cycle, which is atypical relative to the rest. However, despite these cycle characteristics, the natural synchrony of large numbers of eggs within a single egg batch, combined with the relative ease of studying chromosomal configurations, makes this a useful system in studies involving mitotic timing.

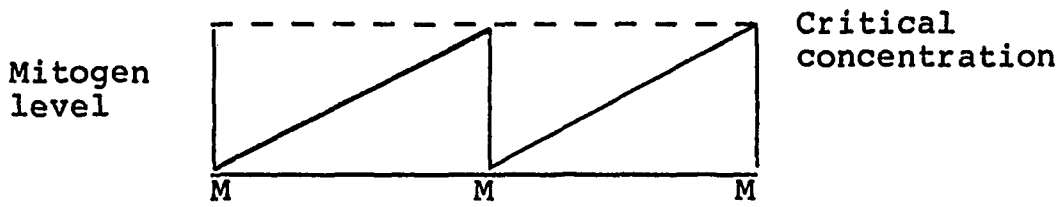
2. Cycle Regulation

The specific control mechanisms involved in cell cycle regulation are poorly understood, although there recently has been a great deal of research into some of the basic biochemical and metabolic events associated with mitosis. As a result, there are several proposed models of cycle regulation in current vogue.

Much of the extensive work on cell cycles involves syncytial masses such as those observed in Physarum, and points to the regulation of cellular transitions by mitogens. In a simplistic sense, a mitogen is a substance or chemical which accumulates and, upon reaching a certain critical concentration, stimulates the cell

to mitosis. There are essentially two models proposed to account for mitogen control of mitosis:

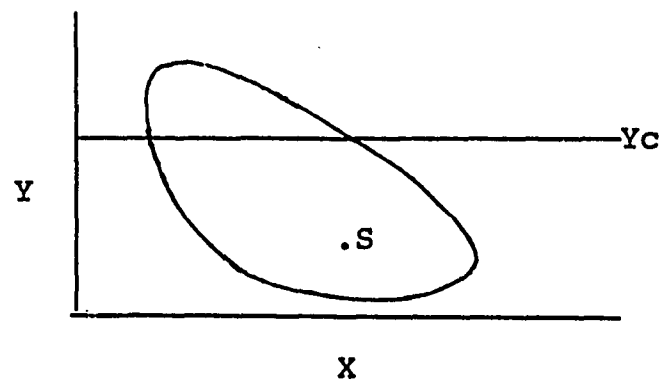
1. The Classical Linear Accumulation Model.



M = Mitosis

In this model, the mitogen accumulates in a linear fashion. When a critical concentration is reached mitosis is stimulated, the mitogen falls back to base levels, and the cycle repeats (Sachsenmaier et al., 1972; Sudberry and Grant, 1975; Fantes et al., 1975).

2. Kauffman-Wille Limit Cycle Oscillator Model.



S = Steady State

In this model there is a sustained limit cycle oscillation of two or more mitogenic substances. All points on the graph represent real possible chemical configurations of X and Y, but only those points within the limits (circle) represent real chemical states in the cycle. Mitosis is reached, in this overly simplified two-mitogen model, when the critical concentration of Y in the appropriate chemical state (Yc) is achieved. S represents the steady state (no mitosis; Kauffman and Wille, 1975).

Several additional theories have been advanced implicating ions as controlling elements of cellular cycles. Calcium, for example, is certainly involved in some aspects of mitotic control, possibly as a control of microtubule assembly and disassembly, or even as a mobilizer of tubulin from cellular pools. pH has been shown to cycle with respect to the mitotic cycle of Physarum (Gerson, 1978). pH may also be involved in Ca fluxes in sea urchins (Epel, 1978), and is thought to be involved in initial Ca release during egg activation.

The release of intracellular calcium at fertilization has been demonstrated by Steinhardt et al.(1977) in the sea urchin, L. pictus, and by Gilkey et al. (1978) in the activating egg of the medaka, Oryzias latipes. In both instances, calcium was

observed photometrically after microinjection of a calcium-sensitive photoprotein, aeqourin. Results in the sea urchin suggest that calcium ion release at fertilization is confined to the subcortical surface. These workers also monitored Ca^{+2} release in response to activation of the unfertilized egg with Ca^{+2} ionophore A23187. This agent has been used in the past as a parthenogenetic activator (Steinhardt and Epel, 1974; Chambers et al., 1974) and stimulates both early phase events (i.e., cortical granule release, fertilization membrane elevation, increased K^{+}conductance) and late phase changes (increases in protein and DNA synthesis). It has been postulated that activation occurs as a result of release of sequestered Ca^{+2}. The work of Steinhardt et al. (1977) confirms that intracellular concentrations increase independently of external calcium concentrations after addition of ionophore to unfertilized eggs.

In the medaka, there is also a rise in intracellular free calcium following fertilization. In this species, however, the free calcium release is propagated as a wave from the point of sperm entry throughout the cell cytoplasm. In addition, the calcium wave was observed to preceed fusion of vesicles with the plasma membrane, thus supporting the idea that it is a calcium

release which triggers cortical granule exocytosis and fertilization membrane elevation (Vacquier, 1975). As in the sea urchin, increases in free intracellular calcium wereindependent of the presence of external calcium. Ionophore activation initiates a calcium wave similar to that stimulated by fertilization, although when large doses of ionophore are used, several waves are initiated from various points. The free calcium wave appears to be self-propagating-that is, it stimulates the release of additional free calcium. The source of this calcium is, as yet, unknown, although some work by Rebhum (1977) involving lanthanium inhibition of cleavage furrow formation suggests that calcium is being stored in association with the plasma membrane, possibly in external surface pools.

In addition to its action as a trigger of cortical granule release, calcium appears to play a much more complex role in the cell cycle. In some vertebrate systems, calcium has been shown to act as a mitogenic agent, and there is much evidence that cAMP is involved in this calcium response (Rebhum, 1977). High levels of calcium stimulate DNA synthesis in cultured 3T3 cells, but not in all other cell lines, suggesting there may be species differences in the mitogen effects of calcium (Rebhum, 1977). The interaction of Ca and

cyclic nucleotides appears to be very complex. In thymic lymphoblasts, cAMP, which is not normally considered to be a mitogenic agent, will act as such in the presence of 0.5 mM calcium, while at 1.5 mM calcium (levels normally mitogenic) cAMP inhibits mitogenesis and DNA synthesis (Whitefield et al., 1973 in Rehbum, 1977).

A calcium mediated ATPase has also been implicated in the mitotic cycle. Mazia et al. (1972) identified this enzyme in mitotic apparatus isolated from echinoderm eggs, and Petzelt (1976) showed initiation of a Ca^{-} – ATPase activity cycle in ammonium activated eggs of S. purpuratus which can be correlated with the chromosome cycle. However, this NH_4 induced cycle lacks an early peak normally present in fertilized eggs. Enzyme activity peaks near the start of chromosome condensation and decreases when nuclear membranes are reformed. Ca - ATPase has been shown to play a role in the Ca-pump of muscle sarcoplasmic reticulum, and is thought to function in controlling cytoplasmic free Ca ion concentrations by causing calcium sequestering or release from muscle cisternae. An extensive endoplasmic reticulum is seen in the cortex of eggs from Xenopus, and it is possible that modified regions of endoplasmic reticulum are acting in a manner analagous to the sarcoplasmic reticulum of muscle cells,

providing a source of Ca^{+2} at activation (Steinhardt et al., 1977). Endoplasmic reticulum has been identified in the spindle of onion root tip cells (Porter and Machado, 1960), and the mitotic gel of sea urchin eggs is also a system of membrane bound vesicles in which microtubules are embedded (Harris, 1978). In addition, the sea urchin aster is composed mainly of microtubules and smooth endoplasmic reticulum (Harris, 1978). These structural implications support the idea that calcium ion concentrations may be controlled in a manner described for muscle tissue-by sequestering and release of ions from the modified portions of endoplasmic reticulum.

A study of the relationship of calcium to cyclic nucleotides, Ca-mediated ATPase and the mitotic apparatus invariable leads to a discussion of the effects of calcium on the microtubular elements associated with the mitotic cycle. For example, increased calcium concentrations cause a breakdown of preexisting microtubules, suggesting that calcium must be lowered by intracellular uptake in order for microtubule formation to occur. It is generally accepted that microtubules are universally associated with chromosomes during cell division. As mentioned previously, the sea urchin aster is composed primarily of microtubules and smooth ER. While the microtubules themselves comprise only a small portion

of the spindle apparatus (the mitotic gel is made up of membrane bound vesicles within which the microtubules are embedded), a close relationship is maintained between these two structures throughout the mitotic cycle (Harris, 1975).

There is much thought that microtubular elements associated with chromosomes are involved in chromosome movement. In this respect microtubules are usually postulated to function as an anchor or attachment site for force producing actin-like filaments (Forer, 1974, review). However, Forer suggests that the force of chromosome movement is provided by actin or myosin-like filaments, but microtubular elements are interacting in a much more active and complex manner to control the rate of movement. The rate limiting step in chromosome movement is microtubule depolymerization, which acts as a damper of rapid movement.

The cytoplasmic microtubular complex (CMTC), representing the association of centrioles, Golgi apparatus and microtubules in interphase cells is first assembled in G_1, and persists until G_2 or early prophase (Fuller et al., 1976). As the cell enters mitosis, the CMTC disappears and shortly thereafter the spindle is formed. If fluorescent labelling of tubular elements during interphase occurs, as the cell enters mitosis and the CMTC breaks down, all fluorescence reappears in the

spindle and associated aster (Brinkley et al., 1975; Fuller et al., 1975), implying there may be a cyclical transition of tubulin from cytoplasmic stores to the mitotic apparatus during the cell cycle (Harris, 1978). If normal interphase eggs are treated with ionophore A23187, Ca ion influx causes breakdown of the extensive CMTC, suggesting that the normal first step in mitosis is Ca ion release (Harris, 1975).

A model proposed by Harris (1978) suggests that mitosis is controlled by the temporal and spatial effects of Ca^{2+} on microtubular assembly and disassembly. This theory postulates that some unknown factor or factors (i.e., pH, critical tubulin levels, cAMP, etc.) stimulate a self-propagated trigger wave of calcium release which initiates at the aster center and radiates outward, causing in its path a change in the state of polymerization of tubulin. While attributing changes in tubulin polymerization to changes in calcium ion concentration, it is still not clear which cytoplasmic microtubular elements are forming mitotic microtubules or what specific event is triggering the initial process. Hence, the basic trigger mechanisms of chromosomal condensation and mitosis remain unknown.

3. Statement of Intent

The objective of this research project was to study the specific effects of two parthenogenetic agents, ammonium chloride and procaine hydrochloride, on the time to first cleavage of sea urchin eggs which have been inseminated post-activation. The ultimate goal of the project is to enhance our understanding of the controlling factors responsible for chromosome condensation and mitosis. It was hoped this experimental approach would aid in the elucidation of the trigger(s) responsible for initiating such cellular events. While the ultimate triggers stimulating the cell to mitosis remain unknown, it is felt that the results presented here will add invaluable insight to our basic understanding of several of the events essential to the programming of cellular cycles.

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CHAPTER 2

PHASE DEPENDENCY OF CLEAVAGE ADVANCE ON THE CHROMOSOME CYCLE IN NH_4Cl ACTIVATED EGGS

INTRODUCTION

In the unfertilized sea urchin egg, metabolic activation in the absence of sperm can be induced by a variety of treatments with specific agents which are termed parthenogenetic activators, although no spontaneous cleavage will occur with treatment by any single agent. Among these are chloroform (Hertwig and Hertwig, 1887), strychnin (Hertwig, 1896 in Harvey, 1909; Morgan, 1900), various salt solutions (Morgan, 1898), hypertonic sea water (Loeb, 1899 in Harvey, 1909), duponal, monogen (Kojima, 1969a), thymol, KCl (reviewed by von Lederbur-Villiger, 1972), the calcium ionophore A23187 (Steinhardt and Epel, 1974), local anaesthetics such as procaine hydrochloride (Vacquier and Brandriff, 1975) and weak bases such as NH_4OH (Loeb, 1913; Mazia, 1974; Mazia and Ruby, 1974) and NH_4Cl (Epel *et al.*, 1974).

The use of these agents to partially activate eggs leads to an uncoupling of the events of the cell cycle in the absence of sperm. To understand this one must first understand the temporal sequence of events unfolding as the unfertilized, biochemically quiescent

egg undergoes metabolic activation after fertilization. A detailed review of the events normally instituted at egg activation, and their sequence of occurrence, can be found in Epel (1975; 1978). Activation is generally broken into two stages - an early phase occurring in the first sixty seconds after sperm contact, and a late phase involving the events occurring at approximately four - five minutes post-insemination (Table 2-1):

A) Activation - Early Phase: As early as three seconds after sperm binding there is a rapid influx of sodium ions. At 20 seconds post-insemination, there is an increase in intracellular calcium ion concentration (probably a result of release of calcium from intracellular stores) and a few seconds later cortical granule exocytosis begins. At this time hydrogen ion efflux begins, mediated by a facilitated sodium-hydrogen transport system (Johnson et al., 1976) and resulting in an elevation of intracellular pH, from 6.84 $\pm$ 0.02 in unfertilized eggs of Lytechinus pictus to 7.02 $\pm$ 0.06 in fertilized eggs (Shen and Steinhardt, 1978). At 34-45 seconds post sperm contact, oxygen consumption increases (Ohnishi and Sugiyama, 1963; Horwitz, 1965; Epel, 1975; 1978), and glucose-6-phosphate dehydrogenase

TABLE 2-1
TEMPORAL SEQUENCE OF DEVELOPMENTAL EVENTS AFTER FERTILIZATION OR ACTIVATION OF SEA URCHIN EGGS

SECONDS	DEVELOPMENTAL EVENT	SPERM	NH_4	PROCAINE	IONOPHORE A23187	REFERENCES
	SPERM BINDING TO EGG SURFACE	+	-	-	-	
1	MINOR INFLUX OF Na IONS MEMBRANE POTENTIAL CHANGE	+	-	?	+	42,43,44
2						
3						
4						
5						
6						
7						
8	Ca^{+2} LIBERATION (FROM INTRACELLULAR DEPOTS)	+	-	?	+	27,45
9						
10						
20	CORTICAL REACTION	+	-	-	+	10,38
	RELEASE OF H^+	+	-	-	+	
	MAJOR Na INFLUX	+	-	-	+	
30	NAD NADP	+	?	?	?	
40	INCREASED O_2 CONSUMPTION	+	-	?	?	5
60	COMPLETION OF FERTILIZATION MEMBRANE	+	-	-	+	
80						
100						
	INCREASED INTRACELLULAR pH	+	+	+	+	19,42
200						
300						
	PROTEIN SYNTHESIS ACTIVATION	+	+	+	+	6,46
400	ACTIVATION OF TRANSPORT SYSTEMS	+	+	+	+	7,12,33,36
500						49
600						
700						
800						
900						
1000						
	PRONUCLEAR FUSION	+	-	-	-	26
	INITIATION OF DNA SYNTHESIS	+	+	+	+	16,29
2000						
3000	FIRST CELL DIVISION IN ACTIVATED EGGS					
4000						
5000						
6000	FIRST CELL DIVISION	+	-	-	-	

is released from intracellular storage depots into the extra-mitochondrial cytosol.

B) Activation - Late Phase: Approximately five minutes post-insemination several biochemical events occur. Protein synthesis increases (Fry and Gross, 1970), various transport systems are activated (Mazia, 1937; Epel, 1972), and there is a rise in potassium ion transport across cellular membranes (Guidice, 1973). In Strongylocentrotus purpuratus, pronuclear fusion and DNA synthesis begin at 20-25 minutes, while mitotic chromosomes appear at approximately 85 minutes (Vacquier and Brandriff, 1975).

That the early and late phase fertilization events represent independent phases of the fertilization response which can be uncoupled is attested to by the fact that eggs are differentially affected by varying the specific agent used to initiate activation (Steinhardt and Mazia, 1973; Epel et al., 1974; Johnson and Epel, 1975). For example, Zucker et al. (1978), using the calcium sensitive photoprotein aequorin, were able to show that parthenogenetic activation by agents such as urea or ionophore A23187 results in internal Ca ion release from intracellular stores,

with its concomitant discharge of cortical granules. Also seen are the later intracellular pH changes and the metabolic events associated with the late phase. These same workers showed that treatment with ammonia did not result in release of intracellular calcium or cortical granule exoctosis, while events of the late phase are initiated, including protein synthesis elevation, activation of various transport systems and DNA synthesis (Mazia and Ruby, 1974; Table 2-1).

Treatment of unfertilized eggs from both S. purpuratus and L. pictus with ammonia stimulates ^{3}H-thymidine incorporation, and eggs treated for an extended period of time display cycles of ^{3}H-thymidine incorporation representative of repetitive S-phases of the cell cycle (Mazia and Ruby, 1974). However, even though successive progressive and regressive phases of the chromosomal cycle are occurring, there does not appear to be aster production and no mitotic apparatus is produced (Mazia, 1974).

Procaine also initiates DNA synthesis in unfertilized sea urchin eggs, but with some slightly different effects when compared with ammonia. These eggs also show an extended S-phase, although in NH_4OH treated eggs DNA synthesis, thymidine transport and chromosome cycles have been reported to continue after removal of the

NH_4OH, while procaine induced events are stopped by procaine removal. In addition to this, eggs treated with procaine produce cytasters which represent condensed, duplicated chromosomes (Vacquier and Brandriff, 1975) and their assembly and disassembly follows the chromosome cycle (Moy et al., 1977).

Treatment with either NH_4Cl or procaine does not appear to irreversibly damage the unfertilized egg. In both instances, unfertilized, treated eggs are capable of being fertilized and will develop into normal larval forms (Brandriff et al., 1975), although care must be taken to use sperm concentrations which reduce the risk of polyspermy. Fertilization can occur at various points within the chromosome cycle, and also at various points within the protein and DNA synthetic cycles. This is accomplished simply, by varying the length of treatment time before sperm addition, and allows one to address the question of phase locking of cleavage rates on these various events. Current evidence indicates that the chromosome cycle is a likely candidate for phase dependency studies. In some preliminary work by Mazia (in Padilla et al., 1974) it was shown that fertilization of eggs activated for 60 minutes with NH_4OH results in condensation of both maternal and paternal chromosomes in the usual 90 minutes (total) required - in other words, the paternal

chromosomes are condensing an hour prematurely. Somehow, addition of the sperm into an egg already into the condensation cycle induces premature condensation of paternal chromosomes. However, it is not clear from this work whether or not the paternal premature chromosome condensation (PCC) is dependent on the phase of maternal condensation, or on other events activated in the egg, such as production of chromosomal proteins required for condensation, activation of various ATPase systems or other enzymes, etc. Mazia suggests that a single 'pervasive switch', implying ions or other small molecules, is triggering paternal PCC. Just what the nature of this switch might be is unknown. Calcium is certainly implicated, possibly as a control of microtubule assembly and disassembly, or even as a mobilizer of tubulin from intracellular pools. Calcium or other ions might also act as limiting factors in the activation of ion dependent ATPases or other Ca-dependent protein systems (calmodulin, for example). pH is likely involved, and may play a role in initial calcium release, suggesting the involvement of dual ionic control systems. The switch may represent accumulation of some unknown mitogenic factor to a critical level which initiates chromosome condensation, or it may be a single surface perturbation/event which is activated at

fertilization to stimulate in a pervasive manner all the metabolic and synthetic events of egg activation.

Recent work by Poccia et al. (1978) indicates that male PCC is indeed paralleling maternal condensation cycles; these workers have shown that in ammonia activated eggs fertilized with sperm concentrations high enough to yield a polyspermic condition (but less than 20 sperm/egg), the male chromatin quickly assumes a physical state similar to the state of maternal chromatin. Density gradient centrifugation of the activated eggs to produce merogons indicates that both nucleated and eunucleated halves are capable of promoting PCC of paternal chromatin, although not to the same degree. However, these workers were able to demonstrate stage specific effects of protein synthesis inhibitors, and have also obtained preliminary results linking a sperm specific histone in the regulation of decondensation of paternal chromatin. All of these results indicate that some cytoplasmic factors are involved in the cycling of chromosomes, but that regulation is probably not completely independent of the female pronucleus.

The complexity of interacting factors involved in the response of eggs to both natural and parthenogenetic activators, and particularly the premature alteration of paternal chromatin states seen upon exposure to

condensed maternal chromatin, whether by sperm/egg interaction or by cell fusion studies (Johnson and Rao, 1971), leads to several interesting questions. Since premature condensation occurs, and all of the events postulated to be required for cell division in this type of system seem to have been met, is the end result an advance in the time required between initial fertilization and first division cleavage? If advanced cleavage is observed, is it also dependent on the phase of maternal chromosome condensation at the time of fertilization? If phase locking of the cleavage advance to the chromosome cycle occurs, how is the advance affected by fertilizing eggs which have been allowed to proceed through a second round of condensation/decondensation? How is division affected in these cells? Are other events of fertilization, such as sperm migration and time to pronuclear fusion, also affected? Finally, is cleavage advance truly phase locked to the chromosome cycle, or is this a manifestation of phase dependency to some other event which is paralleling, at least in part, the chromosome condensation/decondensation cycle? If so, what other maternal systems might be involved, and what role do these systems play in the normal cell cycle?

It is the goal of this research project to attempt to provide answers to some of these questions.

MATERIALS AND METHODS

a. Experimental Animals:

Sea urchins used in this study were collected from two distinct geographical locations. Strongylocentrotus purpuratus and Lytechinus pictus were collected from the San Diego Bay, California. Arbacia punctulata and Lytechinus variegatus were collected at St. Andrews State Park, Panama City Beach, Florida. Animals from the two collection sites were maintained at 10-22^0 C (depending on the species and time of year collected) in refrigerated aquaria containing artificial sea salts. Animals were maintained in a viable state for periods of several months with no noticable loss in egg fertilizability or cleavage synchrony. During this time all animals were fed ad libitum a diet of the naturally occurring seaweeds, Macrocystis sp and Fucus sp.

b. Gamete Collection and Handling:

Gametes were collected by intracoelomic injection of 0.5 M KCL. Sperm were collected 'dry' and stored

on ice until use. Eggs were collected in temperature stabilized Woods Hole formula sea water (MBL-SW; pH 8.0). Eggs were dejellied by acidification of the sea water to a pH of 5.0 - 5.5 with 0.1 N HCl for a period of 1.5 minutes. The pH was readjusted to pH 8.0 by titration with 1.0 M Tris buffer (pH 8.0). Eggs were then washed repeatedly by gravity collection followed by resuspension in fresh MBL-SW. Only batches of eggs showing less than 2.0% germinal vesicles and giving a fertilization response of 95 - 100% were used.

c. Artificial Activation and Fertilization:

In the experiments utilizing ammonia as the parthenogenetic agent, each egg suspension was treated by adding an equal volume of 20 mM NH_4Cl to give a final concentration of 10 mM NH_4Cl (pH8.0) in a 0.1 to 0.5% egg suspension. When procaine was used as the activating agent, the final concentration was either 1.0 or 5.0 mM (pH8.0). Activating agents were removed after appropriate treatment times with two washes in fresh MBL-SW using hand centrifugation.

Fertilization was accomplished by mixing 50 μl of undiluted sperm with 5 ml of MBL-SW and adding 400 μl of this suspension per 40 ml of suspended eggs to give

a final concentration of .01%. Using this sperm concentration, control eggs show 99 to 100% membrane elevation within two minutes, with an average sperm/egg ratio of one.

All experiments were carried out in 50 ml conical centrifuge tubes. Eggs were maintained in suspension with periodic bubbling using pasteur pipets (Epel, 1972).

The experimental procedures for activation of eggs with the two activating agents actually utilized two distinct treatment approaches. In the first series of experiments (Procedure A), eggs of L. pictus and S. purpuratus were inseminated after specific treatment times with the activator removal occurring 30 minutes prior to sperm addition (for example, 80 minutes treatment equals 50 min agent plus 30 min wash). In all instances eggs were fertilized 30 minutes after removal of the activating agents.

In another set of experiments (Procedure B), treatments were begun simultaneously and the length of the treatment wash times were varied. For example, L. variegatus eggs were divided into four conical centrifuge tubes and activated with NH_4Cl as described previously. The total treatment versus wash times were as follows:

1. 30 min NH_4Cl + 70 min MBL-SW

2. 60 min NH_4Cl + 40 min MBL-SW
3. 90 min NH_4Cl + 10 min MBL-SW
4. 100 min MBL-SW (control)

All eggs were fertilized after 100 min total treatment time and monitored for cleavage advance.

d. Controls:

Sperm migration and pronuclear fusion. Eggs treated as described were fertilized and sampled every 5 minutes for a total of 30 minutes. Samples were fixed in Carnoy's fixative with several changes, followed by staining with Leuco Fuschin to enhance visualization of the sperm and egg pronuclei. Male pronuclei in the equatorial plane were scored by measuring the distance migrated from the nearest egg surface (focal depth = approximately 5 μm; see Byrd and Perry, 1980). These eggs were also sampled for cleavage advance.

Polyspermy: Eggs treated as described were fertilized with 400 μl/40ml of the following sperm concentrations (final): .0001%, .001%, .005%, .01%, .05%, 0.1%, 1.0% and concentrated (dry) sperm. Insemination occurred at a time in the cell cycle which results in optimal cleavage advance. Eggs were sampled at

13.5 and 20 minutes post-insemination and fixed in Carnoy's, followed by staining in Leuco Fuschin. Eggs were also sampled in the usual manner for cleavage advance (section e, methods).

e. Sample Collection and Fixation:

In order to assess the chromosomal state of the eggs at the time of fertilization, 1.0 ml samples of eggs were removed and fixed in Carnoy's solution (Ethanol:Acetic Acid, 3:1) overnight. The condensation-decondensation cycle of activated eggs was then monitored by swelling the fixed eggs in 45% acetic acid followed by observation with phase microscopy.

Cleavage in fertilized eggs was monitored by visual observation of live counts supplemented with counts of cleaved eggs in samples collected and fixed at 10 minute intervals post-insemination. The fixative employed in this case was either 2.5% glutaraldehyde (GTA) in sea water or .05% formaldehyde in sea water. Best results were seen when the GTA-SW fixative was used. At least 100 eggs were observed for all samples counted.

RESULTS

a. Phase Dependency of Cleavage Advance:

Eggs from all species studied show a decrease in the time to first cleavage when fertilized following either NH_4Cl or procaine hydrochloride treatment which appears to correlate with the parthenogenetically induced chromosome cycle. Table 2-2 represents a summary of data acquired from all four species studied, where percent condensation is the actual number of eggs from 100 scored showing chromosomes in a condensed state. Cleavage advance is expressed as the difference (in minutes) between the time to cleavage of 50% of the control versus treated eggs, normalized to the total number of eggs cleaved at a time correlating to maximum cleavage in the control group. Normalization of cleavage rates in this manner is necessary to overcome asynchronies in the cleavage rates both within and among different batches of eggs. The use of 50% cleavage times has been used as a criterion for the measure of division timing of single batches of sea urchin eggs in other types of studies involving timing of the mitotic cycle. Rustad (1971) suggests this

Table 2-2. Normalized cleavage advance versus % condensation/decondensation in four species of sea urchin eggs (% condensation is the actual number of eggs in 100 showing condensed chromosomes; C, condensation phase; DC, decondensation phase; and normalized cleavage advance is the time difference between 50% division of control and treated eggs).

A, procedure A; B, procedure B.

Species	Chromosome Condensation (%)	Normalized Cleavage Advance (min)	Activator
S. purpuratus	20 (C)	20	NH_4Cl^A
	80 (C)	30	
	70 (C)	30	
	42 (DC)	30	
(2^{nd} cycle)	58 (C)	15	
			Procaine (1mM)
A. punctulata	0 (C)	5	
	16 (C)	15	
	18 (C)	15	
	53 (C)	35	
	60 (C)	40	
	11 (C)	25	NH_4Cl^A
	32 (C)	65	
	55 (C)	85	
	75 (C)	80	
	84 (C)	90	
L. pictus (1^{st} cycle)	88 (C)	15	NH_4Cl^A
	58 (DC)	52	

Table 2-2. Continued.

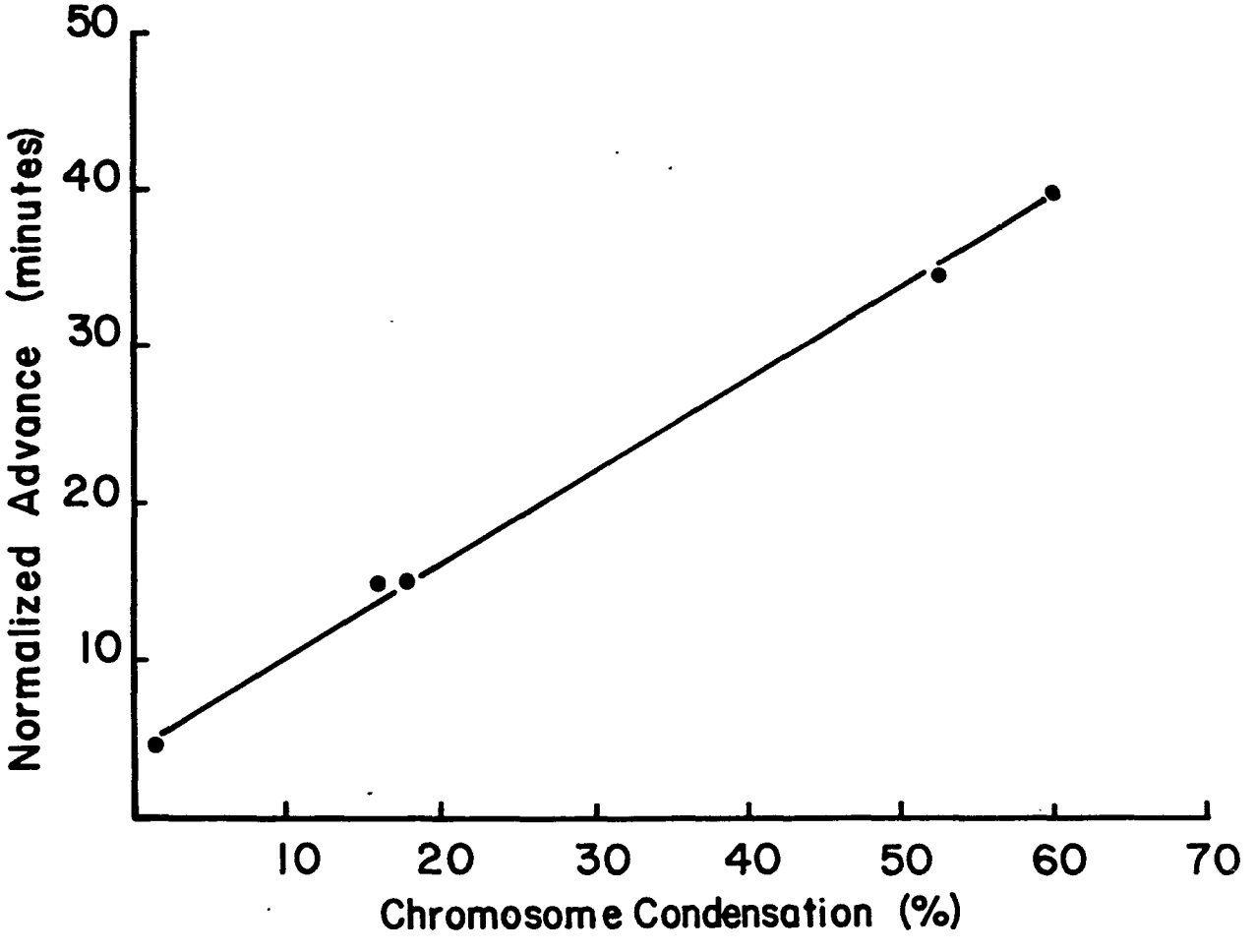
Species	Chromosome Condensation (%)	Normalized Cleavage Advance (min)	Activator
L. pictus			
(2^{nd} cycle)	70(C)	11	NH_4Cl^A
	79(DC)	29	
L. pictus	88(C)	20	NH_4Cl^B
	21(DC)	25	
L. variegatus	88(C)	20	NH_4Cl^B
	93(C)	41	
	73(DC)	41	

criterion is adequate as a measure of mitotic timing in these types of systems where a high degree of synchrony of first division occurs within a population of eggs and very high percents of total cells are dividing.

Chromosome cycle dependency of cleavage advance in eggs from Arbacia punctulata fertilized after treatment with 1 mM procaine hydrochloride (pH8.0) is shown in figure 2-1. Eggs from a single female were divided into five groups and treated with 1 mM procaine for 100, 120, 140, 160, and 180 min, respectively (Procedure A). Eggs in all treatment groups show varying degrees of progressive chromosome condensation at the time of insemination. When cleavage rates are compared to control egg rates, all groups show cleavage advance in direct proportion to the degree of chromosome condensation.

Time dependency of cleavage advance in eggs fertilized after treatment with ammonium chloride (pH 8.0) for a length of time sufficient to produce one complete condensation/decondensation cycle is shown in Table 2-2. Eggs from S. purpuratus were divided into five groups and treated for 60, 90, 120, 150, and 180 min. As in procaine treated eggs, cleavage advance appears to correlate directly with the degree of chromosome

Figure 2-1. Abscissa: percent of eggs scored with condensed chromatin; Ordinate: normalized cleavage advance (in min). Cleavage advance versus chromosome condensation in eggs from A. punctulata fertilized after treatment with 1 mM procaine hydrochloride for 100, 120, 140, 160 and 180 min.

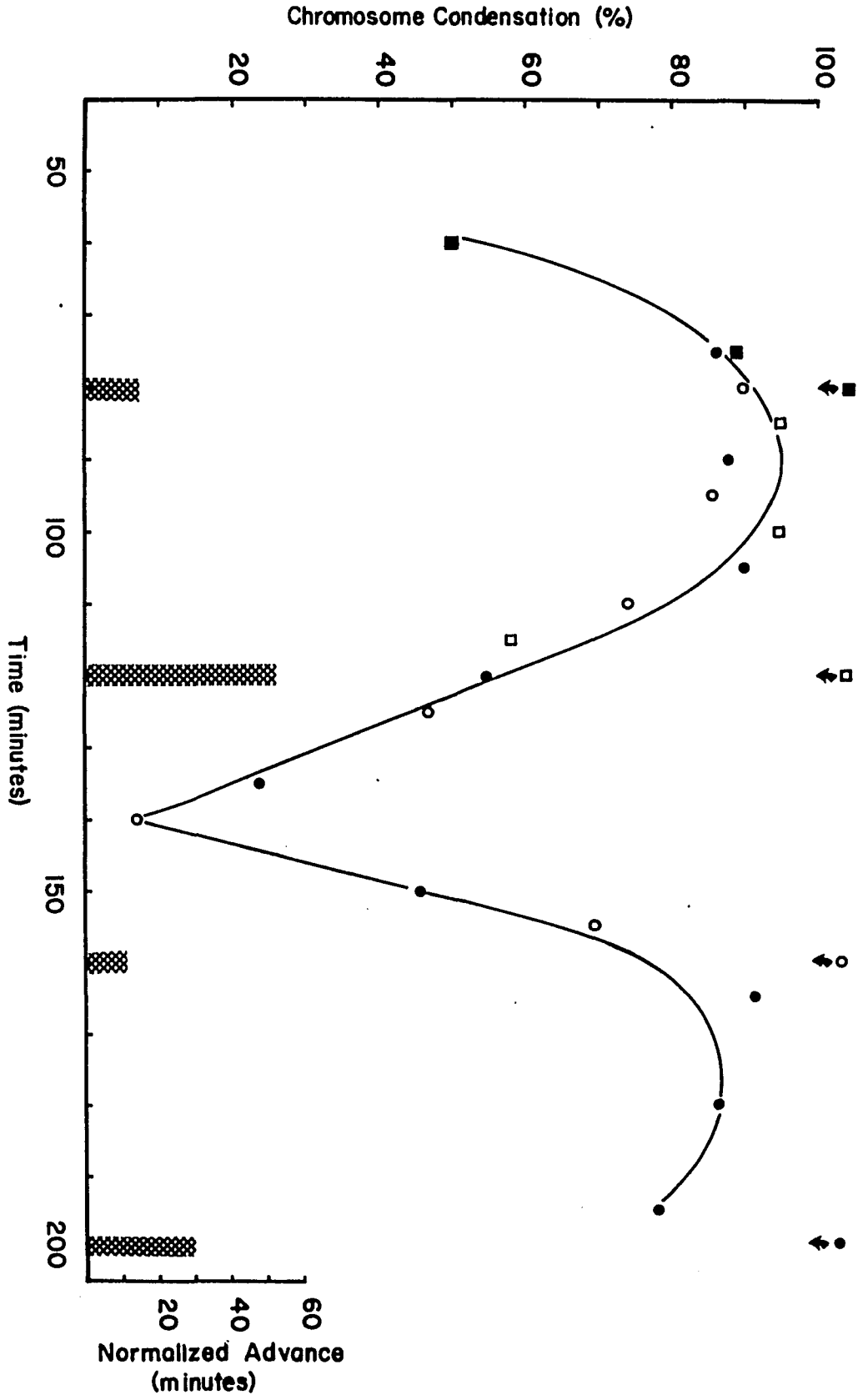

50
40
30
20
10
Normalized Advance (minutes)
10
20
30
40
50
60
70
Chromosome Condensation (%)

condensation, with the greatest advance occurring at maximum condensation, when eggs show chromosomal configurations ranging from metaphase through anaphase.

While cleavage advance appears from the above experiments to correlate directly with the degree of chromosome condensation, it is possible that this advance is associated with some other cellular event that is paralleling the chromosome cycle on a temporal basis. To assess this possibility, eggs were treated for a time length sufficient to produce two consecutive cycles of condensation/decondensation. Eggs from L. pictus were treated with NH_4Cl and inseminated on either side of both condensation cycles (figure 2-2). Eggs fertilized during the second chromosome cycle appear to show the same phase dependency seen when insemination occurs during the first cycle. Eggs fertilized during chromosome condensation of the first cycle show normalized cleavage advance of 15 min (88% chromosome condensation) while eggs fertilized during decondensation of this cycle show an advance of 52 min (58% condensation). Eggs fertilized during chromosome condensation of the second cycle show cleavage advance of 11 min (70% condensed) as compared with an advance of 29 min (79% condensed) when fertilized during decondensation of the second cycle (Table 2-2).

Figure 2-2. Abscissa: time (in min) of NH_4Cl treatment; Ordinate (left): percent of eggs scored with condensed chromatin; Ordinate (right): minutes of normalized cleavage advance in eggs fertilized at various times within the chromosome cycles (arrow).

Eggs from L. pictus were treated with 10 mM NH_4Cl for a total of 200 min. Eggs were fertilized at 80 (■), 120 (□), 160 (○), or 200 (●) min of treatment.


Chromosome Condensation (%)
20
40
60
80
100
50
100
150
200
Time (minutes)
20
40
60
Normalized Advance
(minutes)

When NH_4OH is washed out after pulse treatment of eggs, chromosome condensation and various other events continue to cycle (Mazia, 1974). However, when procaine is washed out, chromosomes continue to condense but do not undergo decondensation, i.e., they do not cycle (Vacquier and Brandriff, 1975). In the experiments performed here, it was necessary to remove the activating agents used from the external medium as a precaution to prevent any effects on fertilization and upon the sperm itself (for example, polyspermy). The effects of washing out NH_4Cl have not been documented, and it was not clear which category of effects would be demonstrated by this activator. Therefore, several experiments involving addition and removal of the activating agents were performed. In addition to clarifying the response of the egg to ammonium wash-out, these experiments provided an ideal means for separating condensation and decondensation phases of the chromosome cycle for study of the individual effects of these phases on cleavage advance.

In the first set of experiments, the effect of washing out procaine hydrochloride on chromosome condensation was studied. Since procaine and NH_4Cl both induce protein synthesis in unfertilized sea urchin eggs, and this event is thought to be a pre-

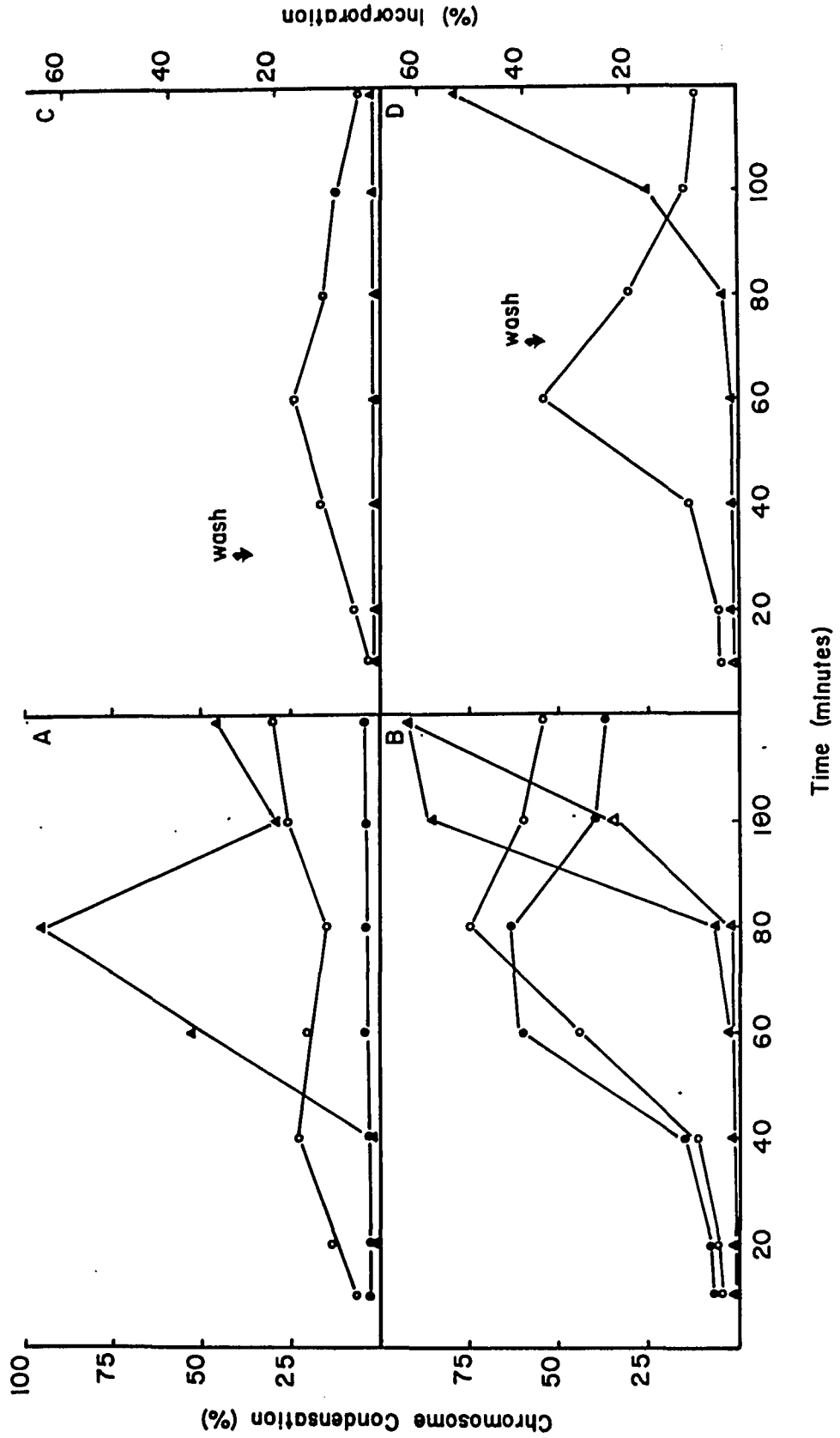
requisite for chromosome condensation (Epel et al., 1974) in sea urchin eggs, I felt the wash out effects on this metabolic system might add insight to the overall problem. Therefore, I studied the effects of activator removal on amino acid incorporation. Eggs from L. pictus were treated with procaine for 30 or 70 minutes, followed by resuspension of eggs in fresh MBL-SW. Samples were taken every 20 min for chromosome condensation analysis and for measurement of % amino acid incorporation for a total of 120 minutes. Results are shown in figure 2-3. Unfertilized control eggs show no chromosome condensation and little amino acid incorporation over the time period sampled (figure 2-3A). Fertilized control (untreated) eggs show a typical pattern of chromosome condensation and amino acid incorporation with first division occurring between 95 and 120 min post-insemination (figure 2-3A). Eggs exposed to continuous treatment with procaine (1 mM or 5 mM, figure 2-3B) show chromosome condensation occurring, but delayed 40 minutes compared to normal fertilized eggs. Amino acid incorporation in both treatments peaks at approximately 80 minutes and then declines. Eggs washed at 30 minutes post-treatment (figure 2-3C) show little chromosome condensation, although amino acid incorporation continues to rise until 60 minutes post-treatment before decreasing. This increase is

Figure 2-3. Abscissa: time (in minutes) of procaine treatment; Ordinate (left): percent of eggs scored showing condensed chromatin; Ordinate (right): percent amino acid incorporation.

Eggs from L. pictus were treated with procaine hydrochloride for 30 or 70 min and sampled at 20 min intervals for chromosome condensation (▲) and percent amino acid incorporation (o). A, controls. Unfertilized, untreated - there is no visible chromosome condensation or significant amino acid incorporation (●). Normal fertilized eggs - chromosomes show maximal condensation at 80 min. Cleavage occurred from 95 - 110 min; B, eggs treated continuously in 1mM (●, % incorporation; ▲, chromosome condensation) or 5 mM (o, % incorporation; ▲, chromosome condensation). Chromosome condensation peaks at 120 min, while percent amino acid incorporation peaks at 80 min; C, eggs treated in 1 mM procaine for 30 min and then washed into MBL-SW. Chromosome condensation is not apparent, although protein synthesis

Figure 2-3. continued.

continues to increase for 60 min before decreasing; D. eggs were treated for 70 min with 1 mM procaine and then washed into MBL-SW. Condensation and percent amino acid incorporation are similar to those of continuously treated controls.


Chromosome Condensation (%)
Incorporation (%)
Time (minutes)
A
B
C
D
wash
wash

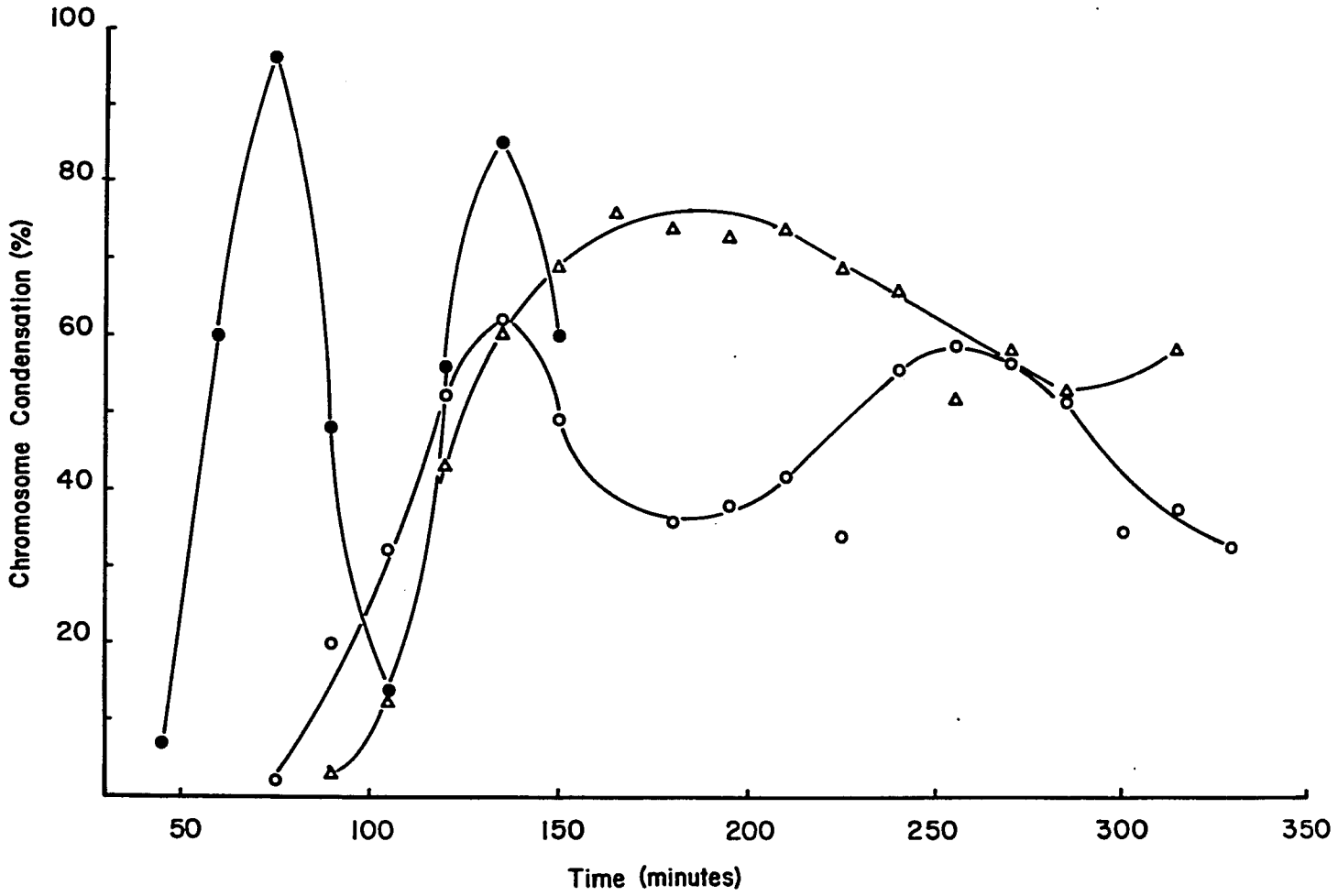
significantly lower than either increase seen in the continuously treated eggs. Eggs washed 70 min after procaine addition show a chromosome condensation pattern similar to that seen in eggs treated continuously. However, amino acid incorporation decreases immediately following procaine removal (figure 2-3D).

The effect of procaine removal on chromosome condensation was studied by monitoring condensation/decondensation cycles before and after procaine removal over a greater time span. In this experiment one group of eggs from S. purpuratus was treated with 1 mM procaine for 330 minutes. A second group was treated with 1 mM procaine for 105 minutes, washed into MBL-SW, and held an additional 225 minutes to a total treatment time of 330 minutes. As seen in figure 2-4, eggs continuously treated with procaine undergo successive cycles of condensation/decondensation. However, when procaine is removed at 105 minutes, eggs continue to condense, but do not show decondensation - chromosomes remained in a condensed state for the duration of the sampling period.

NH_4Cl induced chromosome condensation and amino acid incorporation were also analyzed with respect

Figure 2-4. Abscissa: time (in min) of procaine treatment; Ordinate: percent of eggs scored showing condensed chromatin.

Eggs from S. purpuratus were treated with 1 mM procaine hydrochloride (pH 8.0). ●, normal fertilized eggs; o, eggs treated continuously for 350 min; Δ, eggs treated for 105 min and then washed into MBL-SW and held for an additional 225 min.


100
80
60
40
20
Chromosome Condensation (%)
50
100
150
200
250
300
350
Time (minutes)

to NH_4Cl removal. NH_4Cl was added to eggs from L. pictus and removed at 30 or 70 minutes. Results are shown in figure 2-5. Unfertilized control eggs show no chromosome condensation and no increase in amino acid incorporation over the time period sampled (figure 2-5A). Fertilized control eggs show typical amino acid incorporation and chromosome condensation patterns (figure 2-5A) and normal cleavage in these eggs occurred between 110 and 120 minutes post-insemination. Eggs held continuously in 10 mM NH_4Cl show chromosome condensation and continuously increasing amino acid incorporation (figure 2-5B). Eggs washed at 30 and 70 minutes also show chromosome condensation curves similar to those of continuously treated eggs (figures 2-5, C and D). Amino acid incorporation, while increasing after both 30 and 70 minutes of treatment with NH_4Cl, decreases after peaking at 100 minutes post-activation.

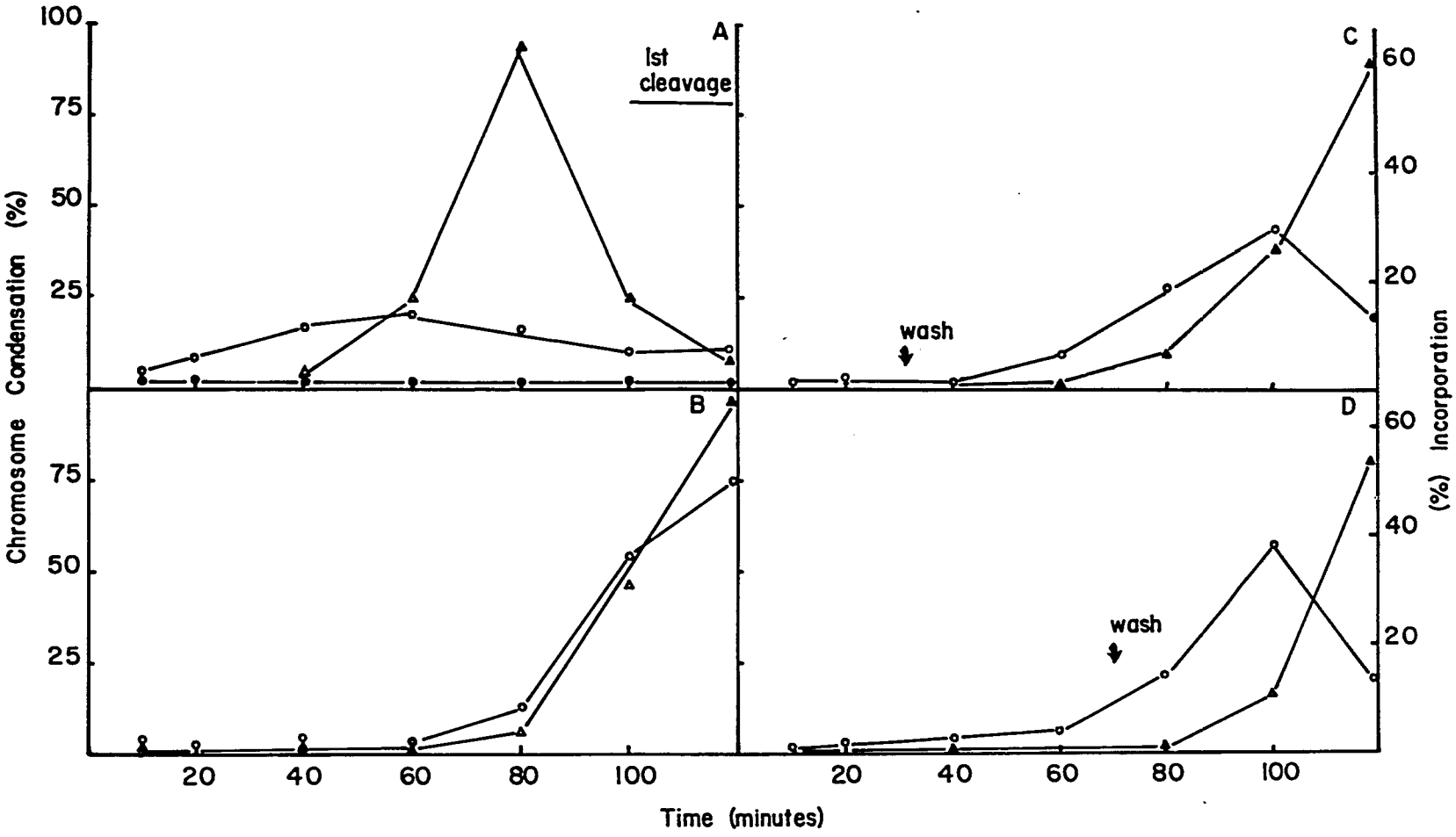
The effects of NH_4Cl removal on condensation/decondensation cycles were studied in both L. variegatus and L. pictus. In both species, NH_4Cl removal does not affect chromosome condensation, but, as in procaine treated eggs, chromosomes do not decon-

Figure 2-5. Abscissa: time (in minutes) of NH_4Cl treatment; Ordinate (left): percent of eggs scored with condensed chromatin; Ordinate (right): percent amino acid incorporation.

Eggs from L. pictus were activated with 10 mM NH_4Cl (pH 8.0). o, percent amino acid incorporation; ▲, chromosome condensation; A, Controls - unfertilized, untreated eggs show no chromosome condensation and no significant amino acid incorporation (●). Normal fertilized eggs show maximal chromosome condensation at 80 min. Protein synthesis reaches a maximal rate at 60 min and cleavage occurred in these eggs at approximately 100-120 min; B, eggs treated continuously in 10 mM NH_4Cl. Chromosome condensation and amino acid incorporation are maximal at 120 min; C, eggs treated with NH_4Cl for 30 min and then washed with MBL-SW. Amino acid incorporation increases for 100 min, and then decreases. Chromosome condensation parallels that of continuously treated eggs; D, eggs were treated for 70 min and then washed into MBL-SW.

Figure 2-5. continued.

Amino acid incorporation increases for 100 min. Chromosome condensation parallels that of continuously treated eggs.


A
1st cleavage
C
wash
B
D
wash
100
75
50
25
60
40
20
Chromosome Condensation (%)
(%) Incorporation
20
40
60
80
100
Time (minutes)

dense but remain in a condensed state (figure 2-6) over the time period sampled. However, if NH_4Cl treatment is continued until the decondensation phase has begun, and then terminated by washing, eggs continue to decondense and even undergo a second round of condensation/decondensation. Decondensation is no longer inhibited by NH_4Cl removal (figure 2-7).

Eggs from S. purpuratus were treated with NH_4Cl and washed at 70 or 90 minutes (figure 2-8). Eggs were sampled for a total of 300 minutes. Continuously treated eggs show continuous cycling of chromosomes. Eggs washed both early in the condensation cycle (70 minute wash) and late in the condensation cycle (90 minute wash) show chromosomes capable of maximal condensation, but no decondensation, verifying that activator removal effects are independent of treatment time within the condensation portion of the cycle in that species also.

In light of the complex nature of events occurring with addition and removal of activating agents, it was deemed necessary to study activator induced cleavage advance with respect to these time dependent variables. Therefore, several experiments employing Procedure B were undertaken.

Figure 2-6. Abscissa: time (in min) of treatment with NH_4Cl; Ordinate: percent of eggs scored with condensed chromatin.

Eggs from L. variegatus were treated with 10 mM NH_4Cl (pH 8.0). o, eggs treated continuously; ●, eggs treated for 70 minutes and then washed into MBL-SW. Chromosomes condensed but do not undergo decondensation.

Figure 2-7. Abscissa: time (in min) of NH_4Cl treatment; Ordinate: percent of eggs scored with condensed chromatin.

Eggs from L. pictus were treated with 10 mM NH_4Cl (pH 8.0). ■,eggs treated continuously; □, eggs treated for 90 min and then washed into MBL-SW. Chromosomes demonstrate successive cycles of chromosome condensation/decondensation.

Figure 2-6

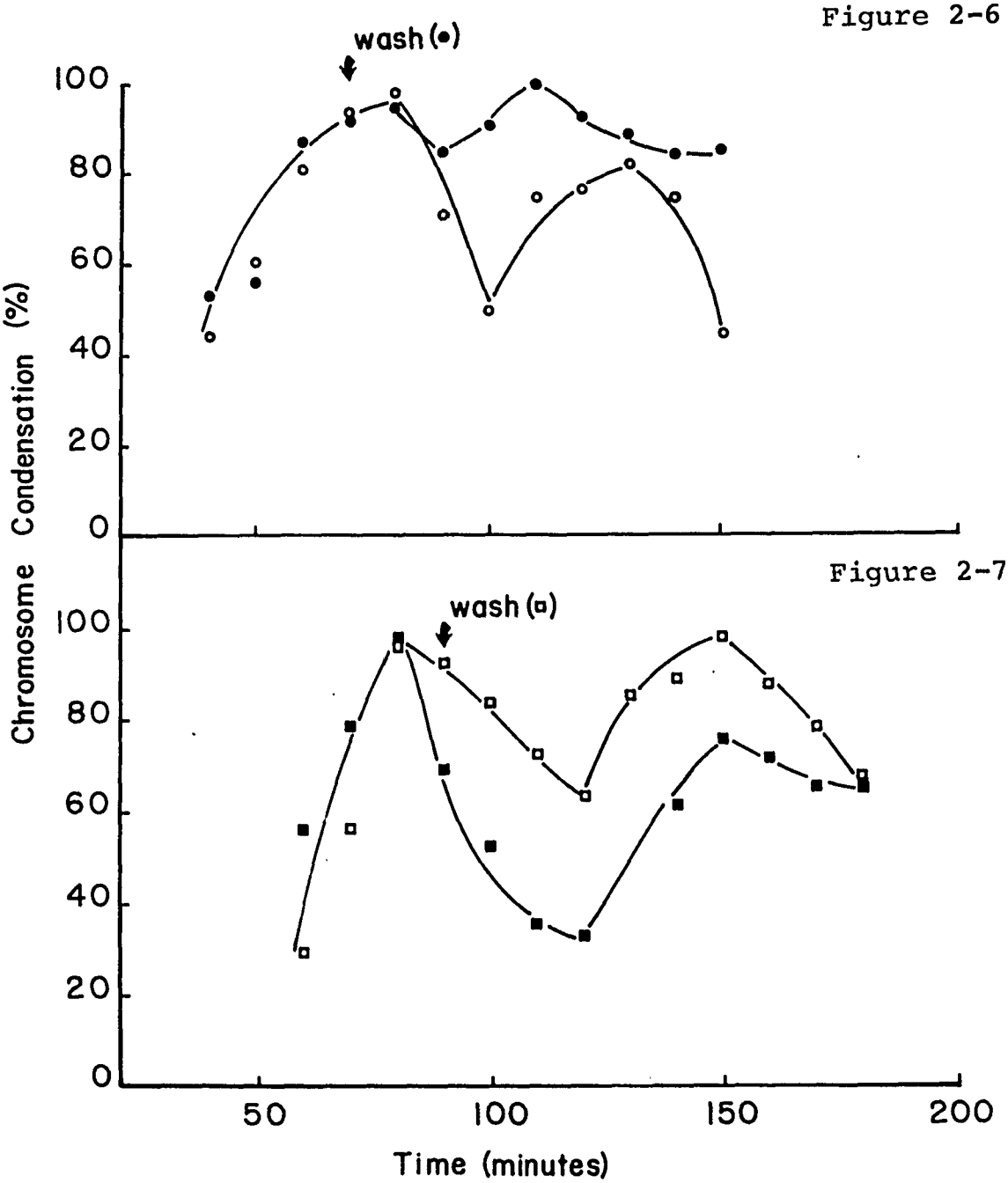
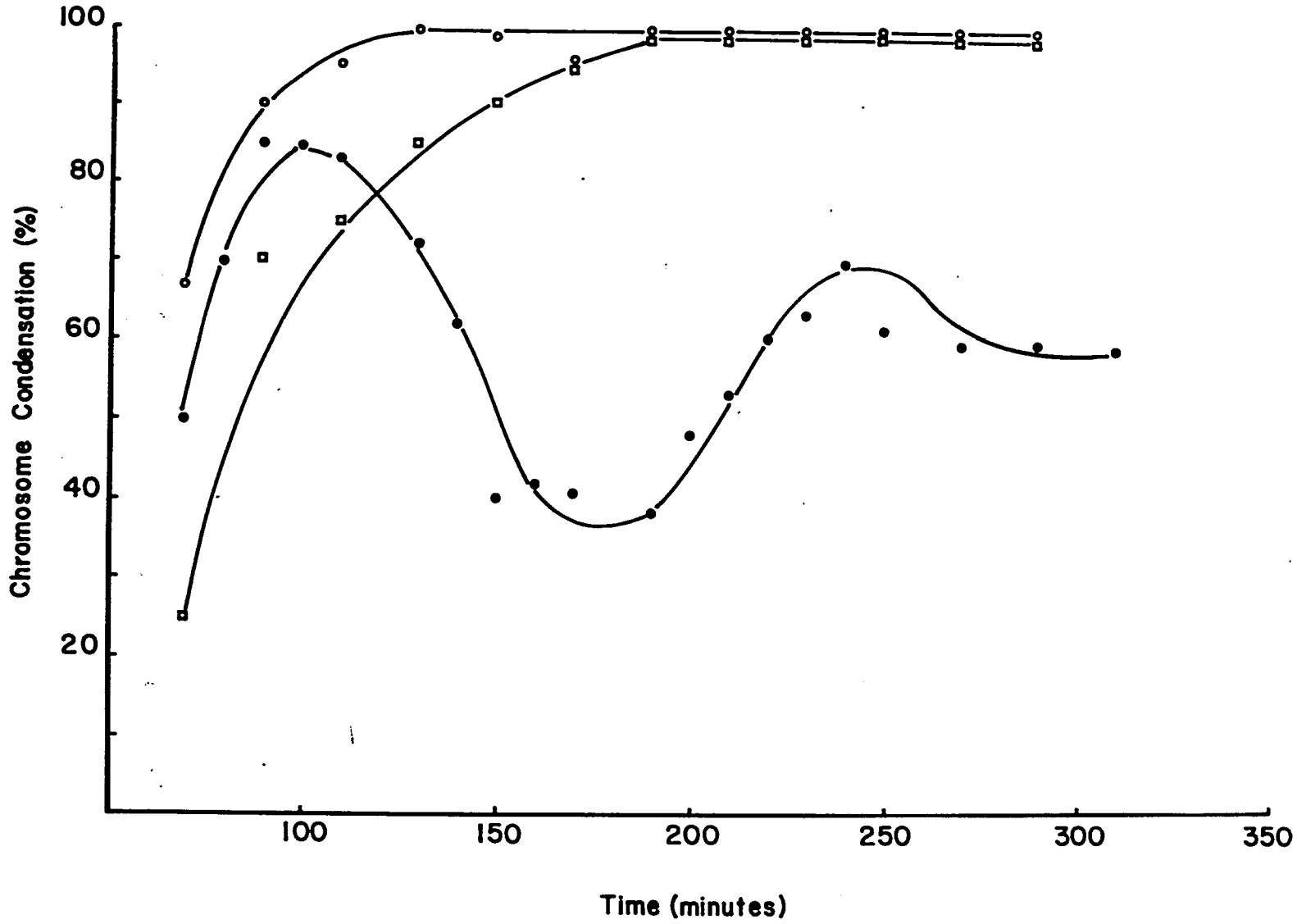


Figure 2-7

Figure 2-8. Abscissa: Time (in min) of treatment with NH_4Cl; Ordinate: percent of eggs scored with condensed chromatin.

Eggs from S. purpuratus were treated for a total of 300 min with 10 mM NH_4Cl (pH 8.0). ●, eggs held continuously in NH_4Cl; ○, eggs treated with NH_4Cl for 70 min and then washed into MBL-SW; □, eggs treated with NH_4Cl for 90 min and then washed into MBL-SW.


100
80
60
40
20
Chromosome Condensation (%)
100
150
200
250
300
350
Time (minutes)

Eggs from L. variegatus were activated with NH_4Cl as described and fertilized after 100 minutes total treatment time. Results are shown in figure 2-9. Eggs exposed to NH_4Cl for only 30 minutes show less than 40% chromosome condensation at any time, and at the time of insemination (100 minutes after NH_4Cl addition) 20% of the eggs contain visibly condensed chromatin. Eggs exposed to NH_4Cl for 60 or 90 minutes show maximum chromosome condensation, although at 100 minutes when eggs are inseminated, eggs treated for 90 minutes appear to be undergoing decondensation (less than 75% remain condensed) while eggs washed at 60 minutes do not (chromosomes in these eggs are 90% condensed at 100 minutes). Eggs treated for 60 and 90 minutes both show normalized cleavage advance of 41 minutes, while eggs treated for 30 minutes show an advance of only 3 minutes (Table 2-2).

Non-cycling and cycling eggs from L. pictus were analyzed for cleavage advance. Eggs treated with NH_4Cl for 80 minutes, washed and inseminated at 140 minutes show approximately 90% condensation of chromosomes at the time of insemination, while eggs held for 130 minutes, washed and inseminated at 140 minutes have completed one cycle of condensation/decondensation and show less than 25% condensation (figure 2-10). Both groups of eggs show significant cleavage advance.

Figure 2-9. Abscissa: time (in minutes) of NH_4Cl treatment; Ordinate (left): percent of eggs showing condensed chromatin; Ordinate (right): minutes of normalized cleavage advance.

Eggs from L. variegatus were treated with 10 mM NH_4Cl (pH 8.0) and fertilized at 100 min. ○, eggs were treated for 30 min and then washed into MBL-SW. Less than 20% of the eggs contain visibly condensed chromatin at the time of insemination. Eggs show a normalized cleavage advance of 3 min (open bar); ▲, eggs treated for 90 min and then washed into MBL-SW show maximal chromosome condensation occurring at approximately 70 min. Eggs show 70% chromosome condensation at the time of insemination, and a normalized cleavage advance of 41 min (cross hatched bar); ●, eggs treated for 60 min and then washed into MBL-SW show greater than 90% chromosome condensation and normalized cleavage advance of 41 min (solid bar).

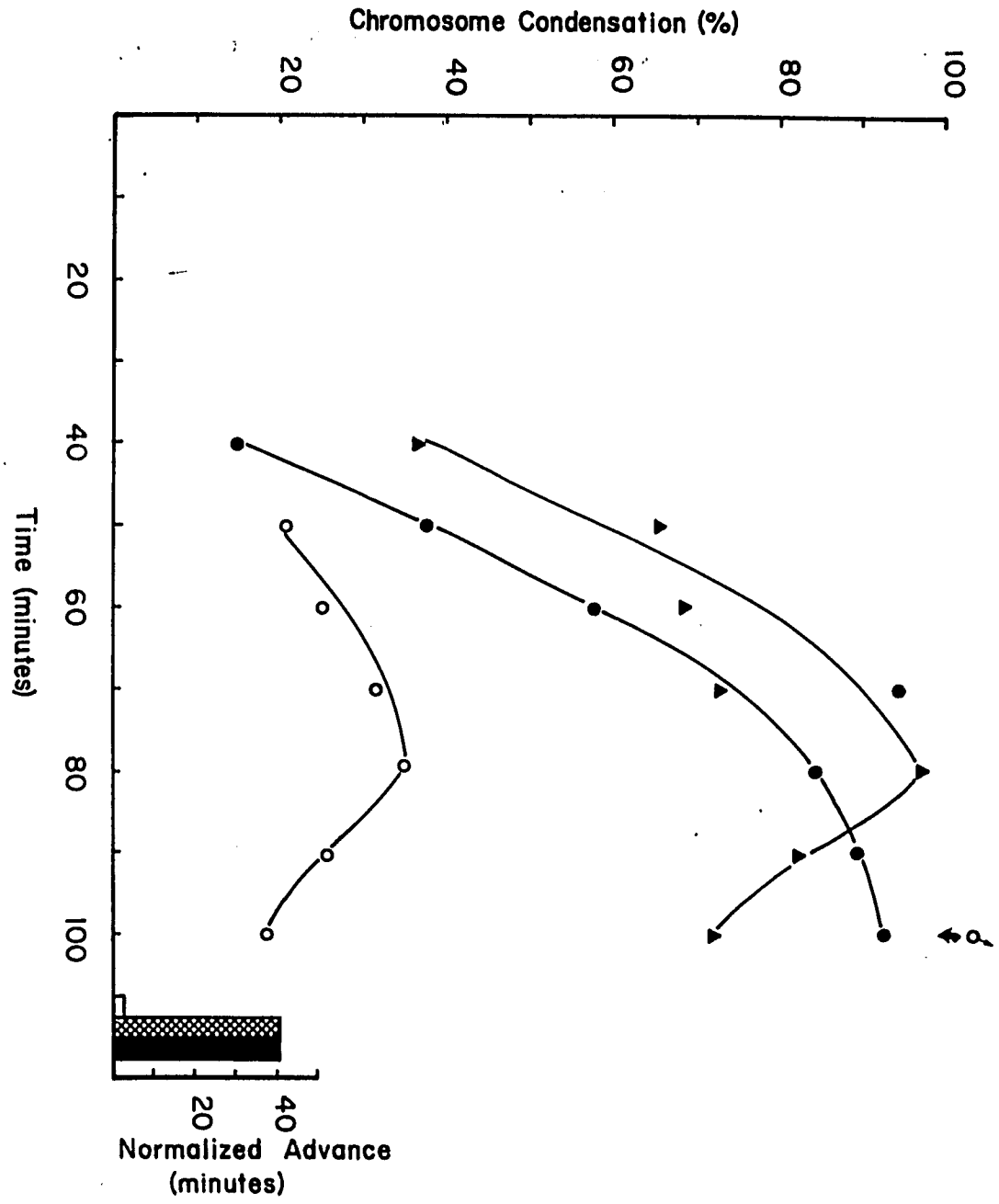
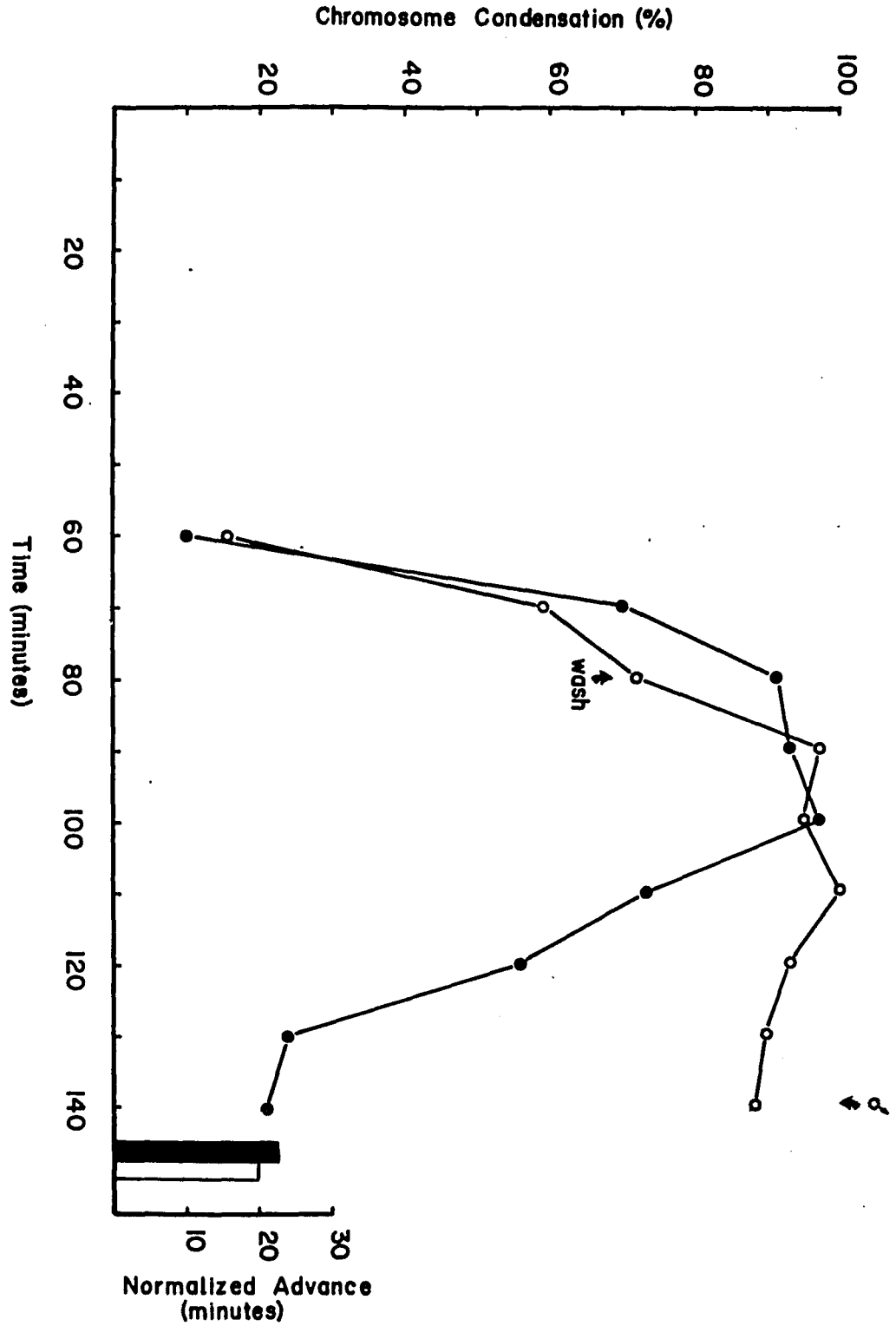

Chromosome Condensation (%)
20
40
60
80
100
Time (minutes)
20
40
60
80
100
20
40
Normalized Advance
(minutes)

Figure 2-10. Abscissa: time (in min) of NH_4Cl treatment; Ordinate (left): percent of eggs scored showing condensed chromatin; Ordinate (right): minutes of normalized cleavage advance.

Eggs from L. pictus were treated with 10 mM NH_4Cl and inseminated at 140 min. ●, eggs were treated for 130 min and then washed into MBL-SW. Chromosomes have undergone decondensation (only 20% of eggs scored contain visibly condensed chromatin). Eggs show a normalized cleavage advance of 20 min (open bar); ○, eggs were treated with NH_4Cl for 80 min and then washed into MBL-SW. Eggs show 88% chromosome condensation and normalized cleavage advance of 25 min (solid bar).


Chromosome Condensation (%)
20
40
60
80
100
Time (minutes)
20
40
60
80
100
120
140
wash
10
20
30
Normalized Advance
(minutes)

Control: Ammonia treated eggs are known to have an increased susceptibility to polyspermy (Mazia, 1974). To eliminate the possibility that cleavage advance was being influenced by the presence of supernumerary sperm as opposed to activator induced alterations in the egg, the effects of polyspermy on cleavage rates and on % cleavage was studied. I found the presence of supernumerary sperm to have no discernable effect on the cleavage advance demonstrated by treated eggs.

Male pronuclear morphology and migration rates were also studied and the premature chromosomal condensation of male chromatin occurred as expected (Poccia et al., 1978). However, there was no increase in the rate of sperm migration or the average time to pronuclear fusion in ammonium activated, inseminated eggs showing cleavage advance. Although it is possible that cleavage advance is being affected by increased rates of events associated with the male pronuclei, indications are that the reverse is true (Poccia et al., 1978) and that the sperm is merely a passive indicator of the maternal condition.

DISCUSSION

Cleavage advance following insemination of sea urchin eggs which have been partially activated by parthenogenetic agents has been documented as a time dependent occurrence. A similar time dependency is shown when sea urchin eggs are treated with agents such as monogen or duponal prior to insemination (Kojima, 1960; 1969). I have shown that when sea urchin eggs are inseminated after pretreatment with the activators NH_4Cl or procaine hydrochloride, a time dependency can be linked phasically to the parthenogenetically induced chromosome cycle. My results indicate that the chromosome condensation/decondensation cycles may be biphasic events with cleavage advance demonstrating linear dependency on the initial phase - that of chromosome condensation. However, the greatest cleavage advance is seen in eggs which have reached maximum percents of condensation (i.e., all chromosomes are in metaphase or later configurations), indicating there may be greater complexity in the relationship between cleavage advance and the chromosome cycle.

Chromosomes in eggs parthenogenetically activated by treatment with NH_4OH seem to show configurations

fairly typical to that of normally fertilized eggs (Mazia, 1974). These NH_4OH treated eggs show repetitive progressive and regressive cycles of condensation. I have observed similar chromosomal configurations occurring in eggs used in this study, where NH_4Cl is used as the activating agent.

The factors normally controlling chromosome condensation (and decondensation) may be studied by the use of parthenogenetic activators. Studies resulting in the premature condensation of interphase type chromatin by fusion of interphase cells with other cells in various stages of meiosis or mitosis have implicated cytoplasmic mediation (Johnson and Rao, 1970, 1971; Barakier and Czolowska, 1977). Since premature chromosome condensation (PCC) occurrs following the fusion of sperm nuclei with eggs containing condensed chromatin the fertilized sea urchin is a useful model system (Poccia et al., 1978). Evidence obtained in these types of studies and in those utilizing anucleate and nucleate ammonia activated eggs and egg fragments verifies cytoplasmic interaction in the control of chromosome condensation(Krystal and Poccia, 1979; Poccia et al., 1978).

In the series of experiments reported in this chapter, no attempt has been made to segregate cytoplasmic influences from chromosomal cycles. Additional

studies are required to more fully answer the question of what factors are controlling cleavage advance. The experiments involving removal of activating agents, which causes the chromosomes to remain in the condensed state in these eggs but the same cleavage advance as compared with eggs which have been allowed to continue to cycle, is evidence that cleavage advance is dependent on the completion of chromosome condensation, but not on decondensation. However, analysis of % amino acid incorporation as a response to activator removal indicates that protein synthesis is not immediately terminated by activator removal. A complete round of protein synthesis occurs in these eggs, although subsequent rounds do not. These findings do not allow one to discount the possibility that cleavage advance is only indirectly linked to the chromosomal cycle through other controlling events. It may even be locked to some other event independently paralleling that cycle. Cleavage advance occurring in eggs inseminated during the first or second round of condensation/decondensation show a similar phase dependency. This suggests that cleavage advance most probably is linked in some dependent manner to the chromosome cycle.

The complexity of cytoplasmic influences on chromosomal cycles is indicated by the apparent phase

dependency on time of activator removal and subsequent effects on condensation. When NH_4Cl removal occurs prior to any decondensation, chromosomes remain in a condensed state. In contrast, when activator removal occurs after decondensation has begun, the chromosomes continue to decondense and even cycle through another complete round of condensation/decondensation. Vacquier and Brandriff (1975) have shown that procaine removal suppresses DNA synthesis. However, Black et al. (1967) found that inhibition of thymidine incorporation in fertilized sea urchin eggs by protein synthesis inhibitors has little or no effect on S-1, but subsequently effects S-2. This suggests that factors required for condensation and division are produced at least one duplication cycle prior to their use. Possibly this is why activator removal during the decondensation phase of the chromosome cycle has no effect on the subsequent condensation cycle. This suggests that the critical factors necessary for decondensation are relatively stable and allow two (or more?) chromosome condensation/decondensation cycles. I have demonstrated that NH_4Cl removal prior to maximum condensation uncouples the condensation and decondensation phases of the cycle. This finding is unusual, as generally once a cell is "committed' to S it will proceed through mitosis (Mazia, 1974). Perhaps, as Vacquier

and Brandriff (1975) suggest, it is chromosome decondensation which is the essential factor controlling entrance into S.

It is possible that effects on the chromosome cycle may be directly attributed to the resulting internal pH changes caused by activator removal, and not to interruptions in the cyclical synthesis of critical molecules needed for initiation and completion of chromosomal cycles. NH_4Cl is known to induce increased interior alkalinity in sea urchin eggs. The mechanism of NH_4Cl induced pH increases involves the attainment of equilibration of charged versus uncharged species of ammonia as a function of its passive distribution between the cell exterior and interior (Winkler and Grainger, 1978). A rapid equilibration occurs when eggs are exposed to external sources of NH_4Cl or procaine (Winkler and Grainger, 1978). However, the time required for recovery of internal pH to the unfertilized level when activators are removed is presently unknown. Preliminary indications are that recovery may take 30 minutes or more (discussed in Chapter 4). Any apparent time dependent effects of NH_4Cl or procaine removal must take into consideration the time course of action of activator addition and/or removal on internal pH, and on inhibition of various metabolic processes.

In summary, cleavage advance in sea urchin eggs fertilized after activation with agents such as NH_4CL or procaine hydrochloride demonstrates a phase dependency on the parthenogenetically induced chromosome cycle. This cycle appears to be biphasic, consisting of distinct condensation and decondensation phases which can apparently be uncoupled by the removal of the activators. The effects of activator removal are time dependent, implying that once the cell has passed a critical point, it is committed to chromosome decondensation. These results imply an inherent complexity in the cycling of chromosomes that heretofore has not been recognized. Cleavage advance was demonstrated to be independent of chromosome decondensation by the use of these uncoupling methods, but it is not clear whether cleavage advance is directly phase-locked to the chromosome cycle, or only indirectly linked through other cyclic events occurring within the activated egg.

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CHAPTER 3

CYCLOHEXIMIDE INHIBITION OF CHROMOSOME DECONDENSATION AND CLEAVAGE ADVANCE IN NH_4CL ACTIVATED SEA URCHIN EGGS

INTRODUCTION

Ammonium chloride induces metabolic activation of sea urchin eggs, and acts to initiate the late-phase series of fertilization events without initiating the early phase changes associated with sperm or ionophore activation (see Chapter 2, Table 2-1). Two of the metabolic events triggered by NH_4Cl, chromosome condensation and protein synthesis, show independent threshold levels to stimulation by NH_4Cl. The chromosome condensation cycle has been shown to have at least a partial dependency on protein synthesis (Epel et al, 1974).

Chromosome condensation/decondensation cycles are responsive to the addition and removal of NH_4Cl in in a time dependent manner (Chapter 2). When unactivated eggs are treated with 10 mM NH_4Cl at pH 8.0 and then washed and incubated in fresh MBL-SW (minus NH_4Cl), the eggs continue to exhibit chromosome condensation at the same time as eggs held continuously in NH_4Cl. However, chromosomes do not undergo decondensation as seen in continuously treated eggs. But, if eggs have entered the decondensation phase of the cycle when the NH_4Cl

is removed, chromosomes continue to decondense and even undergo a subsequent cycle. These effects are not due simply to the immediate pH changes associated with NH_4Cl removal, as preliminary indications are that internal pH may take up to 30 minutes to return to untreated levels following NH_4Cl removal (see Chapter 4).

We have shown that when NH_4Cl treated eggs are fertilized, a first division advance occurs relative to control eggs which is dependent on the duration of NH_4Cl treatment (Chapter 2, see also Kojima, 1960; 1969). Cleavage advance appears to be dependent on the parthenogenetically induced chromosome condensation. In eggs that have chromosomes held in a maximally condensed state by NH_4Cl removal, subsequent fertilization results in cleavage advance. Since the condensation-decondensation phases are uncoupled, this suggests cleavage advance is not directly dependent on chromosome decondensation.

Eggs treated with NH_4Cl for 15 minutes show increases in protein synthesis rates, but not to the same level as continuously treated eggs. There is no cleavage advance or chromosome condensation in these eggs. In order to establish metabolic phase specific dependencies of cleavage advance, it is necessary to study that advance with respect to the chromosome cycle in

eggs where protein synthesis has not been activated. To determine the respective roles that chromosome condensation and protein synthesis play in cleavage advance, eggs were activated with 10 mM NH_4Cl in the presence of the protein synthesis inhibitors, cycloheximide and emetine.

In addition, chromosome condensation cycles in eggs which are NH_4Cl - cycloheximide treated in ion substituted sea water were monitored in an attempt to isolate possible ionic regulators.

Calcium and pH are postulated to play critical roles in the initiation and modulation of protein synthesis (Winkler et al, 1980). It is possible that separation of the ionic factors involved in initiation of protein synthesis and chromosome condensation may shed light on the nature of NH_4Cl induced chromosome condensation. For this reason I have included chromosome cycle studies involving activation of eggs by the Ca^{2+} Ionophore A23187. This ionophore has been shown to activate early Ca^{2+} changes associated with the cortical reactions of sea urchin eggs, and the late phase series of events (Steinhardt and Epel, 1974).

MATERIALS AND METHODS

The sea urchins, Lytechinus variegatus, were collected from St. Andrews State Park, Florida. Strongylocentrotus purpuratus and Lytechinus pictus were collected from San Diego Bay, California. Animals were maintained in refrigerated aquaria from 10-22° C, depending on the species and the time of year collected. Animals were fed ad libitum a mixture of the seaweeds, Macrocystis sp. and Fucus sp. Gametes from these three species were collected and processed by the methods previously described (Chapter 2).

a. Protein Synthesis Inhibition in Activated Eggs:

To inhibit protein synthesis, eggs were activated in 10 mM NH_4Cl at pH 8.0 according to previously described methods (Chapter 2) while in the presence of either cycloheximide or emetine.

To determine the minimum effective concentration of cycloheximide, eggs were activated in various concentrations of this inhibitor ranging from 0.1 mM to 10 mM. At periodic intervals, 2 ml samples of treated

eggs were removed and pulse labelled with either ^{14}C- or ^{3}H-labelled amino acids (Epel, 1972). The percent amino acid incorporation was determined by the method of Johnson and Epel (1975).

To determine the reversibility of cycloheximide inhibition of protein synthesis, eggs were activated with NH_4Cl while in the presence of cycloheximide (2-10 mM). At 60 minutes, cycloheximide was removed by washing the eggs into 10 mM NH_4Cl minus cycloheximide. Eggs were sampled before and after treatment.

b. Inhibitor Effects on Chromosome Condensation and Cleavage Advance:

Eggs were treated simultaneously with NH_4Cl and cycloheximide or emetine (5×10^{-5} M) for a time length sufficient to produce maximum chromosome condensation in NH_4Cl (minus inhibitor) treated controls. Eggs were then washed with MBL-SW to remove both the activator and the inhibitor. Eggs were inseminated approximately 30 minutes after washing. Aliquots of eggs were removed at 10 minute intervals prior to insemination, and fixed in Carnoy's as described (Chapter 2). Eggs were then scored for chromosome condensation. Egg samples were also removed at 10 minute intervals post-insemination, fixed in formaldehyde-sea water,

and observed for cleavage. The normalized cleavage advance was calculated as described in Chapter 2.

c. Activation in Ion Substituted Sea Water:

Eggs were activated with NH_4Cl or Ca^{2+} Ionophore A23187 in the following different ion substituted sea water combinations at pH 8.0.

1. 10 mM NH_4Cl in MBL-SW (control).

2. 10 mM NH_4Cl in Na or Ca free (^{o}Na,^{o}Ca) sea water. Choline chloride was used to replace NaCl. Ca free sea water was chelated with 5 mM EGTA.

3. 10 mM NH_4Cl in Na free (Choline substituted) sea water.

4. 2.5 micromolar Ionophore A23187 in MBL-SW (control).

5. 2.5 micromolar Ionophore A23187 in Na free (choline substituted) sea water.

RESULTS

a. Protein Synthesis Inhibitors:

Cycloheximide treatment of activated eggs from L. variegatus indicates that concentrations exceeding 5 mM reduce protein synthesis levels by greater than 95% over levels obtained in NH_4Cl activated eggs in this species. Similar results are seen with eggs from S. purpuratus. However, in L. pictus, 5 and 10 mM cycloheximide reduces protein synthesis to only 75%-88% of NH_4Cl activated controls. The inhibitory effects are completely reversible in all species. When cycloheximide treated eggs are washed into 10 mM NH_4Cl-SW minus cycloheximide, protein synthesis rates increase to 100% of the levels seen in NH_4Cl treated eggs. Protein synthesis levels reached a maximum rate when monitored for % amino acid incorporation 30 minutes after cycloheximide removal in L. variegatus.

b. Chromosome Condensation:

Chromosome condensation could not be completely inhibited in NH_4Cl activated eggs using the protein synthesis inhibitors, cycloheximide and emetine.

Treatment of NH_4Cl activated eggs from S. purpuratus with 1.0, 2.5 and 5.0 mM cycloheximide are shown in figure 3-1. No chromosome condensation was seen in eggs treated with 10 mM cycloheximide. However, treatment of eggs with 5 mM cycloheximide delays condensation relative to condensation seen in eggs treated with lower concentrations of inhibitor, which are identical to controls. In contrast, no chromosome condensation is observed in eggs of L. pictus at cycloheximide concentrations of 5 mM or greater.

Similar experiments were performed using emetine in place of cycloheximide. Emetine has been reported to inhibit 95-99% of incorporation of amino acids into proteins in sea urchins when used in concentrations exceeding 5×10^{-5} M (Hogan and Gross, 1971; Epel et al., 1974). When eggs from S. purpuratus are activated with 10 mM NH_4Cl in the presence of 5×10^{-5} M emetine, chromosome condensation occurs, but chromosome decondensation does not. The block of decondensation is not reversed when eggs are washed with 10 mM MN_4Cl 80 minutes after activation to remove the inhibitor (figure 3-2), implying emetine may not have reversible effects.

Results of activation of eggs from S. purpuratus by NH_4Cl in the presence of 4 mM cycloheximide in ion

Figure 3-1. Abscissa: time (in minutes) of NH_4Cl-cycloheximide treatment; Ordinate: percent of eggs scored showing condensed chromatin. .

Effects of cycloheximide on NH_4Cl induced chromosome condensation in S. purpuratus. ●, 1.0 mM cycloheximide; ○, 2.5 mM cycloheximide; ■, 5 mM cycloheximide; □, NH_4Cl - no cycloheximide.

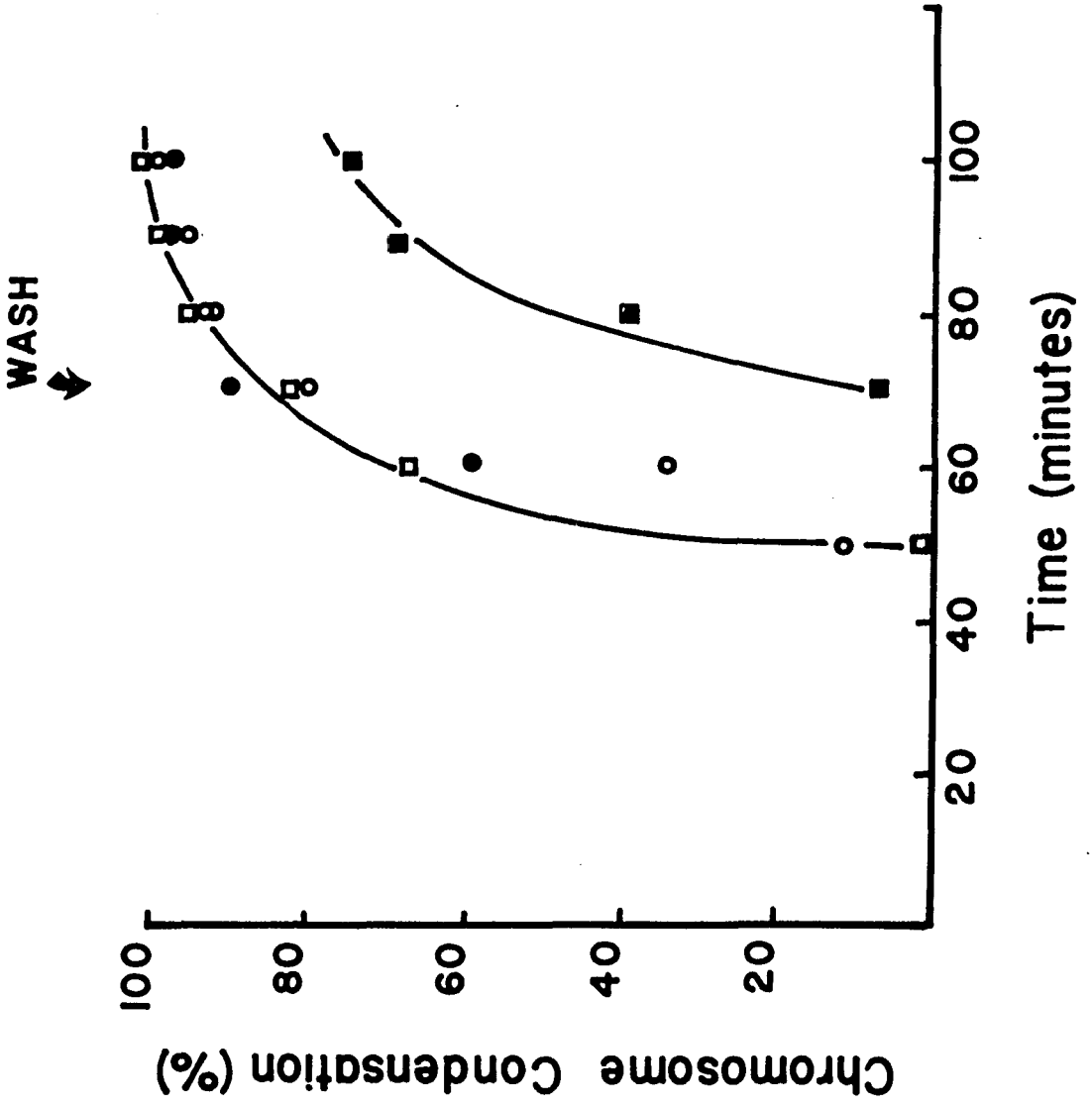
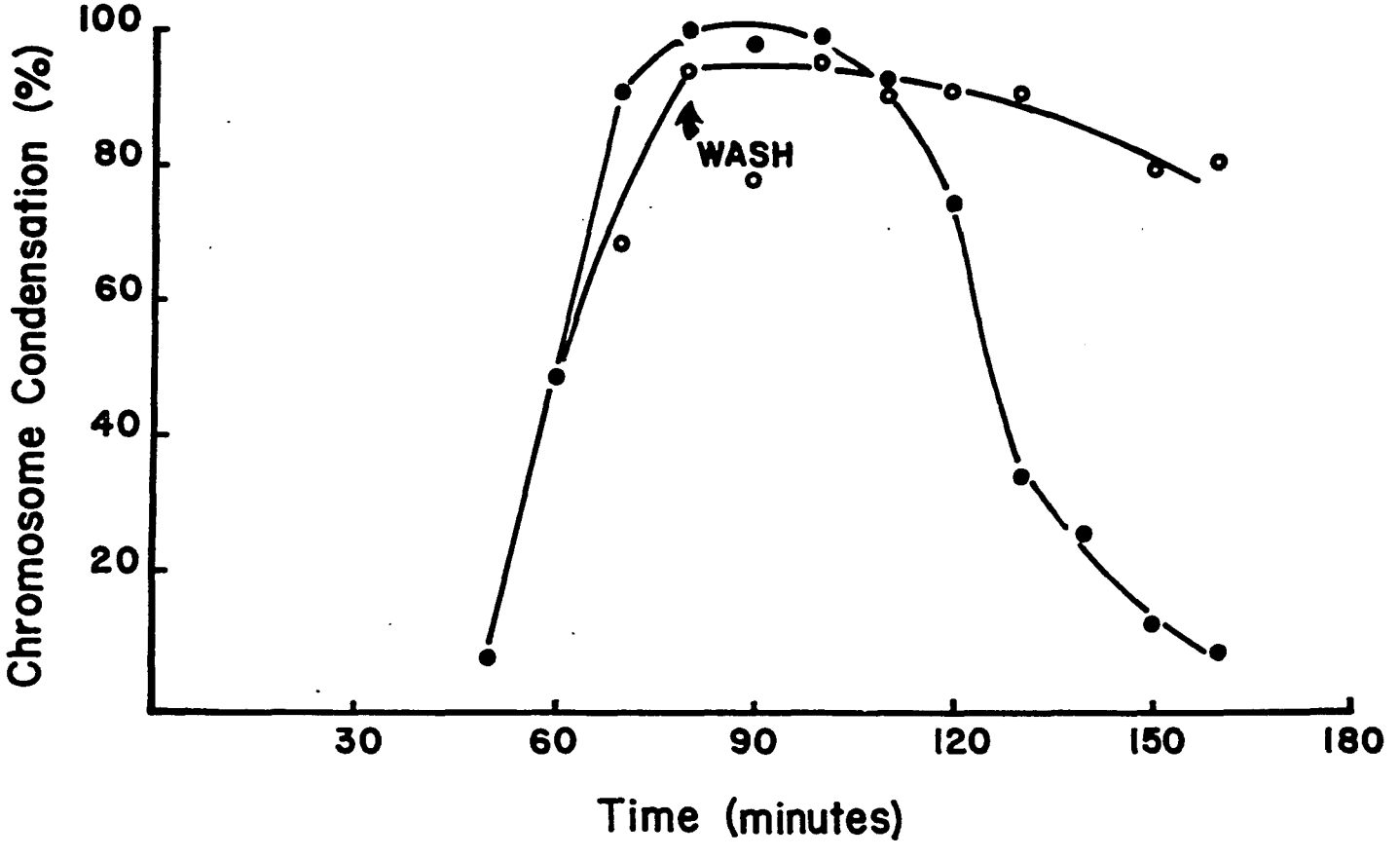

WASH
Chromosome Condensation (%)
100
80
60
40
20
20
40
60
80
100
Time (minutes)

Figure 3-2. Abscissa: time (in minutes) of NH_4Cl-emetine treatment. Eggs were washed into 10 mM NH_4Cl sea water (pH 8.0) at 80 minutes; Ordinate: percent of eggs scored showing condensed chromatin.

Effects of emetine on chromosomal cycles in S. purpuratus. ●, 10 mM NH_4Cl (pH 8.0); ○, 10 mM NH_4Cl with 5×10^{-5} M emetine.


Chromosome Condensation (%)
100
80
60
40
20
WASH
30
60
90
120
150
180
Time (minutes)

substituted sea water are shown in figure 3-3. In these experiments, eggs were treated continuously for 200 minutes. As in emetine treated eggs, condensation is not affected by this level of cycloheximide, but decondensation is delayed over the time period studied. Similar results are seen when eggs are activated by Ionophore A23187 while in the presence of 4 mM cycloheximide.

c. Effects of Inhibitors on Cleavage Advance:

The effects of 5×10^{-5} M emetine on cleavage advance in NH_4Cl activated eggs from S. purpuratus are shown in figure 3-4. Eggs were treated for 70 minutes, then washed into MBL-SW. Sperm were added at 100 minutes, and the normalized cleavage advance determined. No cleavage advance was observed in emetine treated eggs, while NH_4Cl activated eggs show a normalized advance of 29 minutes over untreated controls. Chromosome condensation is not affected by treatment with emetine in these experiments.

Treatment of eggs from S. purpuratus for a time length sufficient to produce a second cycle of condensation is shown in figure 3-5. Eggs were treated for 150 minutes, and washed into MBL-SW. Insemination occurred at 180 minutes post-activation. Eggs activated

Figure 3-3. Abscissa: time (in minutes) of NH_4Cl treatment; Ordinate: percent of eggs scored showing condensed chromatin.

Effects of cycloheximide on NH_4Cl induced chromosome cycles in S. purpuratus. Δ, 10 mM NH_4Cl with 4 mM cycloheximide in normal sea water; □, 10 mM NH_4Cl in normal sea water (minus cycloheximide).

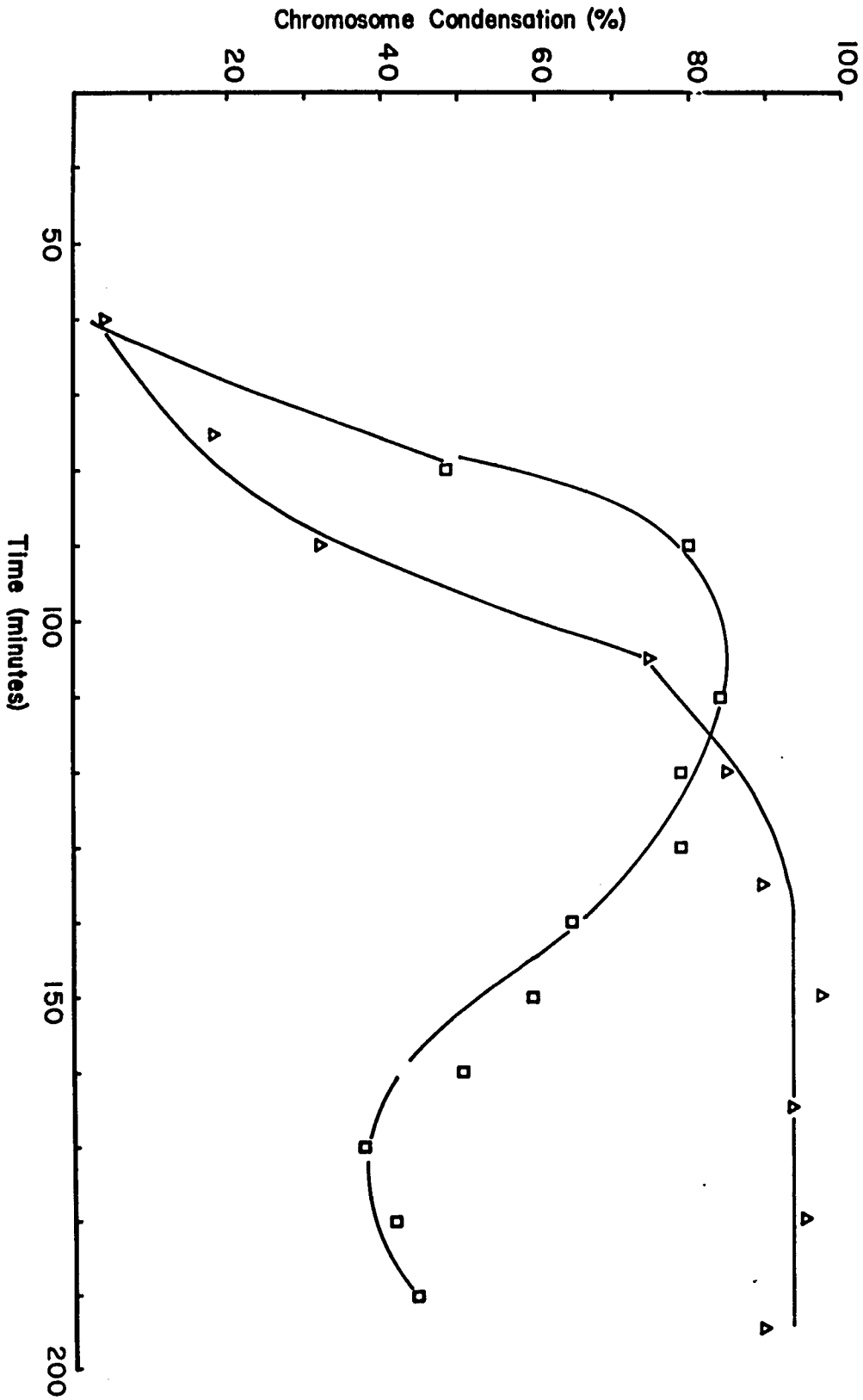

Chromosome Condensation (%)
20
40
60
80
100
Time (minutes)
50
100
150
200

Figure 3-4. Abscissa: time (in minutes) of NH_4Cl, emetine treatment. Eggs were washed in normal sea water at 70 minutes; Ordinate (left): percent of eggs scored showing condensed chromatin; Ordinate (right): minutes of normalized cleavage advance in control eggs when fertilized at 120 minutes (cross hatched bar).

Effects of emetine on chromosome cycles and cleavage advance in S. purpuratus. ●, 10 mM NH_4Cl; ○, 10 mM NH_4Cl with 5×10^{-5} M emetine.

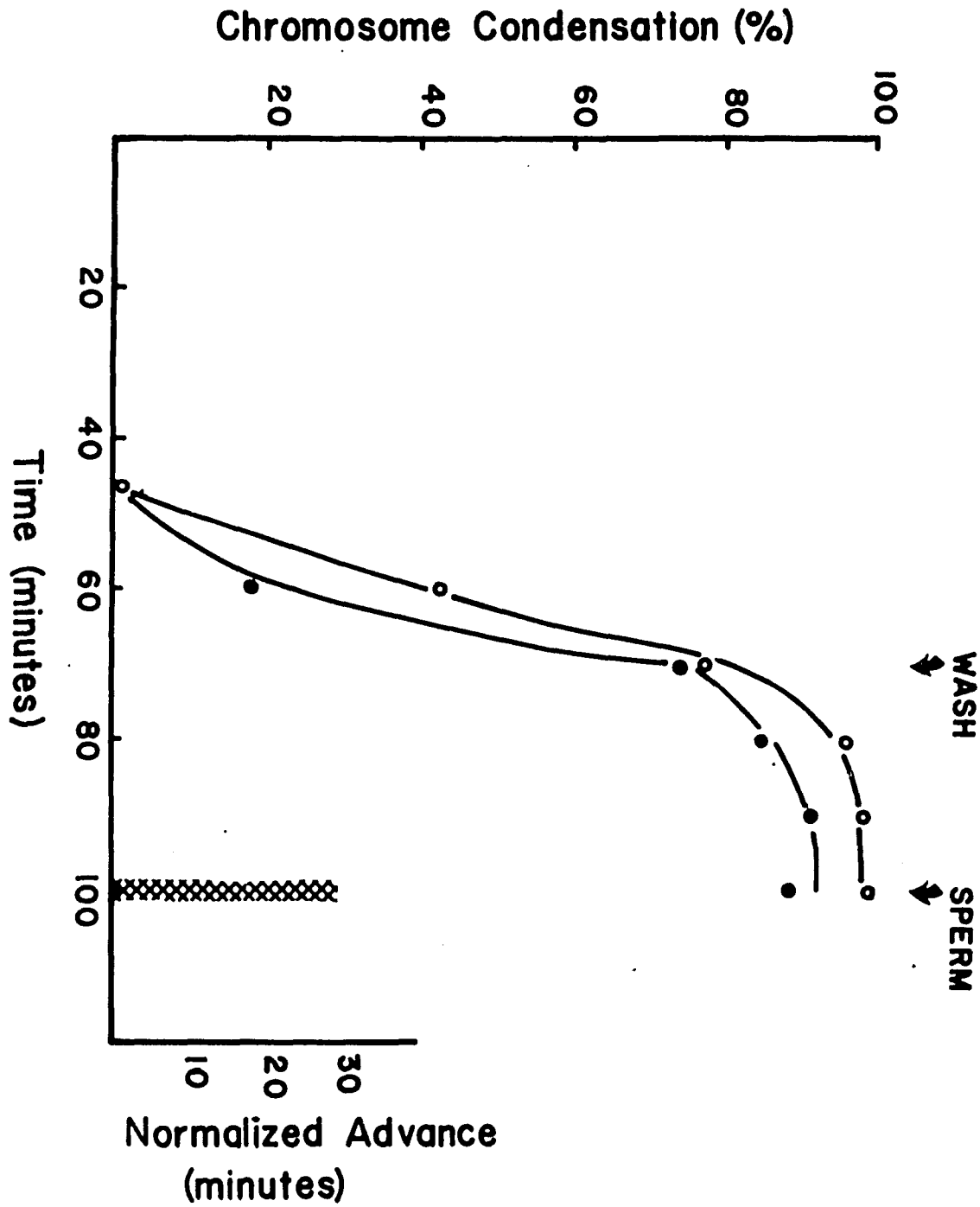
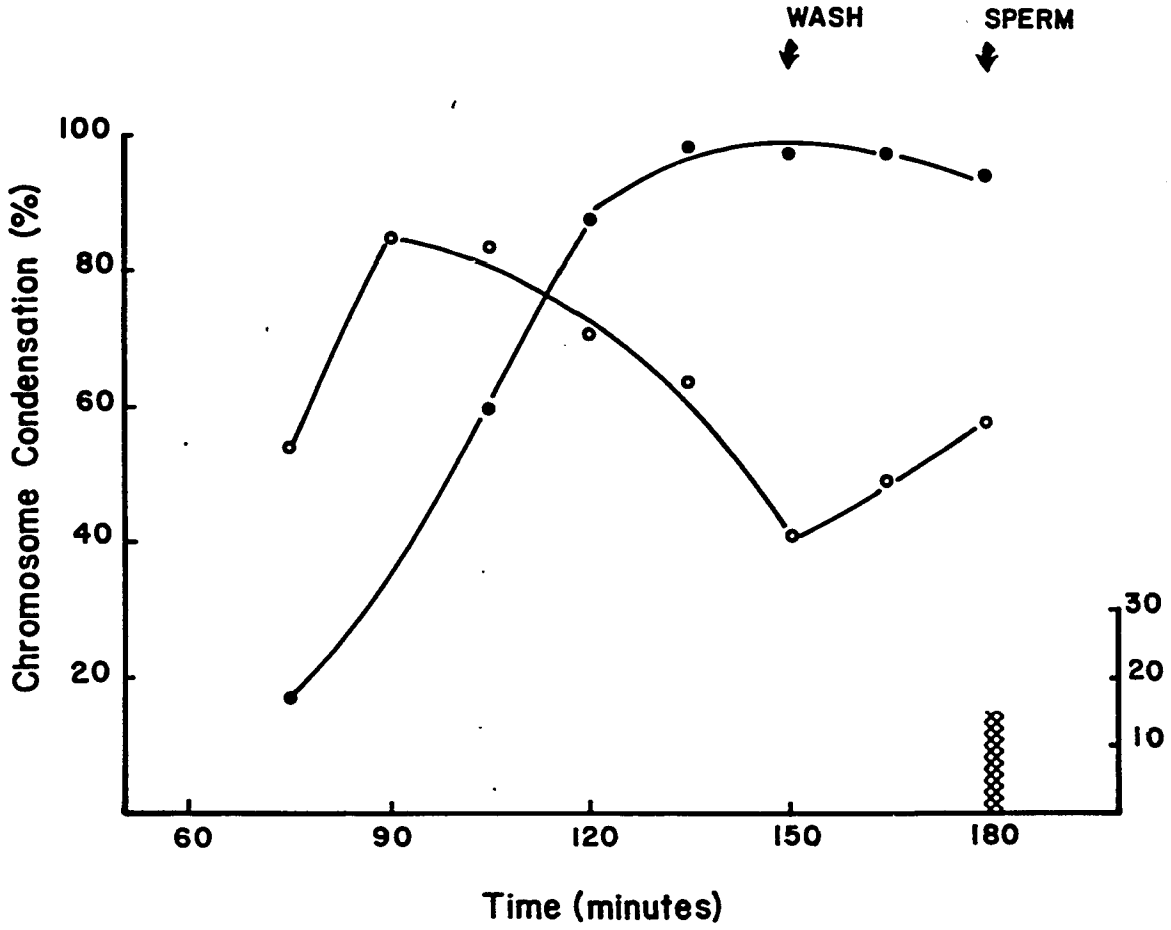

Chromosome Condensation (%)
20
40
60
80
100
Time (minutes)
20
40
60
80
100
WASH
SPERM
10
20
30
Normalized Advance
(minutes)

Figure 3-5. Abscissa: time (in minutes) of NH_4Cl, cycloheximide treatment. Eggs were washed in MBL-SW at 150 minutes; Ordinate (left): percent of eggs scored showing condensed chromatin; Ordinate (right): minutes of normalized cleavage advance in control eggs.

Effects of cycloheximide on NH_4Cl induced chromosome condensation and cleavage advance when eggs are fertilized at 180 minutes. O, 10 mM NH_4Cl (cross hatched bar); ●, 10 mM NH_4Cl with 5 mM cycloheximide (solid bar).


WASH
SPERM
100
80
60
40
20
Chromosome Condensation (%)
30
20
10
60
90
120
150
180
Time (minutes)

with 10 mM NH_4Cl in the absence of protein synthesis inhibitors show a normalized cleavage advance of 15 min. Eggs activated in the presence of 5 mM cycloheximide do not demonstrate cleavage advance. As in previous experiments, chromosome condensation is not affected, but there is no chromosome decondensation.

d. Activation in Ion Substituted Sea Water:

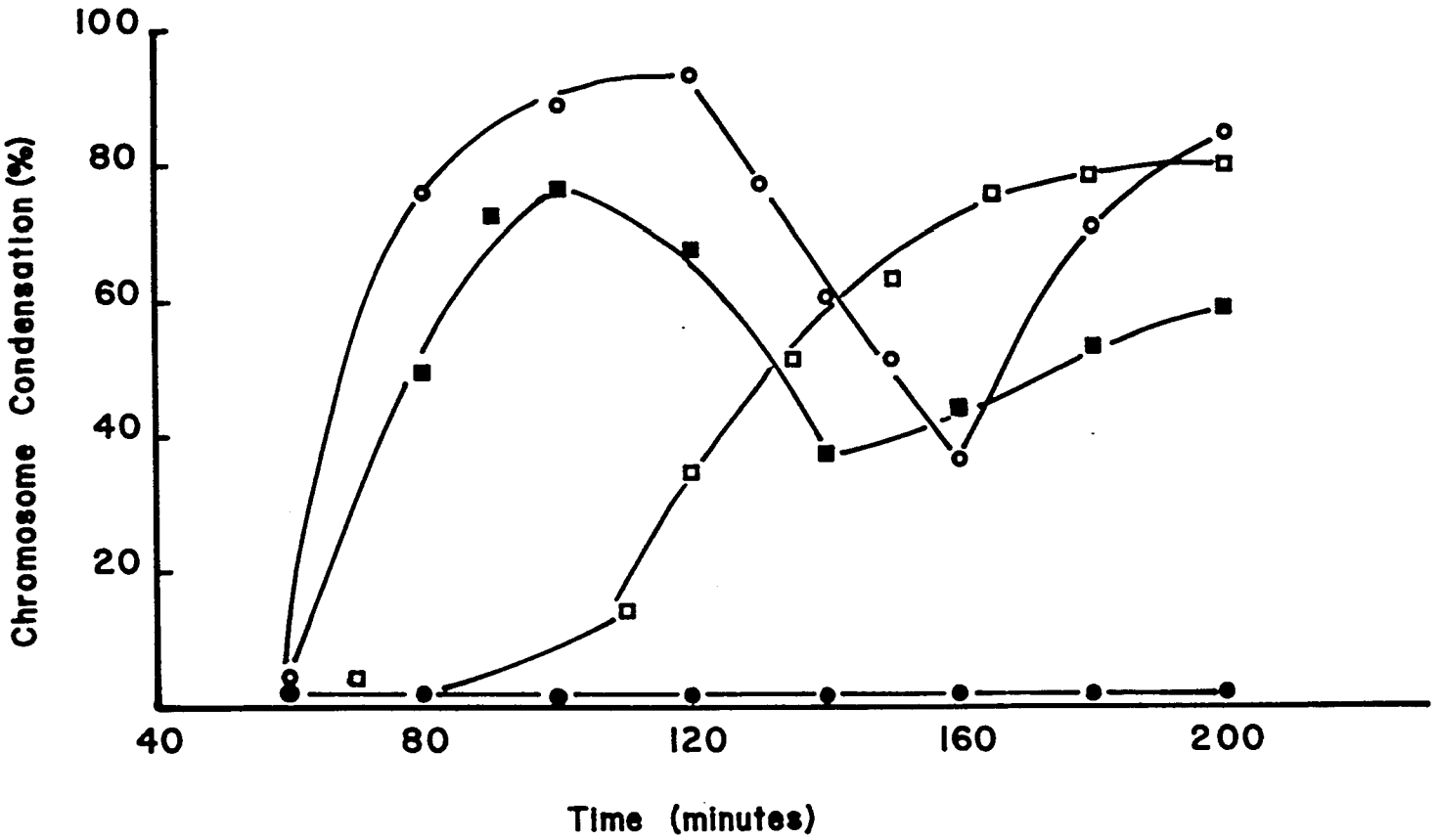
To determine ion dependencies of chromosome condensation, several experiments involving NH_4Cl and Ionophore A23187 activation in ion substituted sea water were performed.

1. Activation of eggs by 2.5 μM Ionophore A23187 in normal MBL-SW (^{+}Na) initiates typical condensation/decondensation cycles in S. purpuratus (figure 3-6). However, activation in ^{o}Na (choline substituted) sea water does not initiate chromosome cycles, although fertilization membrane elevation has occurred, indicating that the early Ca^{++} events are being activated in these eggs.

2. To insure that no external Ca fluxes occurred in NH_4Cl activated eggs, eggs from S. purpuratus were activated in ^{o}Na, ^{o}Ca (EGTA chelated) sea water. Normal chromosome cycles are initiated in these eggs (figure 3-6).

Figure 3-6. Abscissa: time (in minutes) of treatment; Ordinate: percent of eggs scored showing condensed chromatin.

Effects of ^{o}Na and ^{o}Ca on chromosome condensation. ○, 2.5 μM Ionophore A23187 in normal (^{+}Na) sea water; ●, 2.5 μM Ionophore A23187 in ^{o}Na sea water; □, 10mM NH_4Cl + 4 mM cycloheximide in ^{o}Na, ^{o}Ca (EGTA chelated) sea water; ■, 10 mM NH_4Cl in ^{o}Na, ^{o}Ca (EGTA chelated) sea water.


100
80
60
40
20
Chromosome Condensation (%)
40
80
120
160
200
Time (minutes)

3. In an attempt to uncouple protein synthesis, and transient Ca^{++} fluxes, from NH_4Cl induced increases in intracellular pH, eggs from S. purpuratus were activated by NH_4Cl in ^{O}Na, ^{O}Ca sea water in the presence of 4 mM cycloheximide. As seen in figure 3-6, chromosome condensation is initiated, but no decondensation occurs over the time period studied.

DISCUSSION

In this study I found that inhibition of protein synthesis by use of metabolic inhibitors prevents the cleavage advance seen in eggs fertilized after pretreatment with NH_4Cl. I also present evidence that chromosome condensation is independent of protein synthesis. However, in the absence of protein synthesis, chromosome decondensation does not occur.

Cycloheximide has been shown to induce specific patterns of cell cycle progression delay in Physarum which are dependent upon the stage of the cell cycle when the inhibitor is administered (Scheffey and Wille, 1978). Cycloheximide has been shown to alter circadian rhythmicity in several species, including Euglena (Feldman, 1976), Acetabularia (Karakashian and Schweiger, 1976) and Aplysia (Rothman and Strumwasser, 1976). Nakashima et al. (1980) have demonstrated that cycloheximide inhibition of circadian clock function in cycloheximide resistant mutants of Neurospora is due to inhibition of 80 S protein synthesis, and not

to cycloheximide effects on other cellular mechanisms.

Addition of cycloheximide to sea urchin eggs which have been activated by NH_4Cl does not completely inhibit chromosome condensation but delayed condensation and prevented chromosome decondensation. Following fertilization, no cleavage advance was observed in these eggs. Results presented here indicate that cleavage advance can be uncoupled from chromosome condensation. Our findings suggest that mitotic timing of the first division cycle in sea urchin eggs is controlled, at least in part, by production of proteins which may or may not be involved in chromosome decondensation. It is possible, however, that cycloheximide or emetine inhibition of cleavage advance in sea urchins is a result of inhibitor action at sites other than cytosolic protein synthesis. Hogan and Gross (1971) have reported that emetine, at 10^{-4} M, inhibits DNA synthesis in sea urchins. However, experiments conducted by Wagenaar and Mazia (1978) demonstrated that 10^{-3} M emetine, while effectively inhibiting protein synthesis in sea urchins, has no effect on DNA synthesis or replication. These workers also report that inhibition of chromosome condensation and mitosis in normally fertilized eggs is phase specific. They noted that proteins produced

early in the cell cycle are required for completion of chromosomal cycles and mitotic timing. Their observation is in agreement with my finding that cycloheximide inhibits cell cycle advance. My observation that chromosome decondensation was dependent on protein synthesis suggests that factors influencing entry into S are proteins involved in the control of chromosome decondensation (see also Vacquier and Brandriff, 1975; Chapter 2).

Activation of eggs by Ca^{+2} Ionophore A23187 in Na-free sea water results in intracellular calcium release, but no increase in intracellular pH or protein synthesis (Winkler et al., 1980). In my experiments, when eggs are activated by Ionophore in Na-free sea water, no chromosome condensation occurs, suggesting that increased intracellular Ca^{+2} alone is insufficient to induce chromosome condensation. However, chromosome condensation does occur when eggs are activated with A23187 in MBL-SW in the presence of cycloheximide. These data indicate that either chromosome condensation is dependent on the Na^{+} induced pH shift, or that cycloheximide is not inhibiting protein synthesis sufficiently to inhibit condensation.

When eggs are activated with NH_4Cl in Na, Ca-free (chelated) sea water, no Ca^{+2} fluxes

should occur, but increased intracellular pH and protein synthesis are triggered (Winkler et al.,1980). When eggs are activated under the above conditions, I observed typical condensation cycles, thus verifying that chromosome condensation is not dependent on Ca^{+2} fluxes. When cycloheximide is added to the Ca, Na-free activation medium, chromosome condensation occurs, but decondensation does not. It appears that NH_4Cl affects chromosomal condensation via its effects on internal pH, and only influences decondensation via its effects on protein synthesis. Internal pH has been shown to cycle with respect to the cell cycle in Physarum (Gerson, 1978) and in sea urchin eggs (Epel, 1978; see also Chapter 4). Protein synthesis, in turn, has been shown to be regulated via a dual ionic control mechanism involving the interaction between internal Ca fluxes and internal pH (Winkler et al., 1980). Internal pH may act as the switch initiating protein synthesis and chromosome condensation. Ca interacts to regulate protein synthetic rates (Winkler et al., 1980). Proteins, in turn, are postulated to provide the factors required for chromosome decondensation and cleavage advance (this paper). Possibly critical levels of decondensation proteins represent the in-

teracting component that drives the cell to mitosis. Perturbations in any component could cause phase shifts in mitotic timing, thus explaining both cleavage advance in NH_4Cl treated eggs, and inhibition of advance in cycloheximide treated eggs.

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CHAPTER 4

NH_4Cl INDUCED SURFACE CHANGES IN PARTHENOGENETICALLY ACTIVATED SEA URCHIN EGGS

INTRODUCTION

Studies of sea urchin egg topography following fertilization indicate that eggs undergo profound alterations in surface structure. Among these are the normal fertilization events encompassing cortical granule exocytosis and leading to fertilization membrane elevation and hardening (see Giudice, 1973; Czihak, 1975 for reviews). At the plasma membrane there are also additional morphological changes occurring. These changes in plasmalemma surface topography can be viewed with the scanning electron microscope, and indications are that the surface undergoes a series of structural changes throughout cell development (Sanger and Sanger, 1980).

Tegner and Epel (1973) have demonstrated in nine species of sea urchins and in two additional species of sand dollars, that the unfertilized egg is characterized by uniform arrays of surface proturbances representing microvilli. In Strongylocentrotus purpuratus, microvilli of unfertilized eggs are approximately 0.5μ in

length and show a density of about 10 per square micrometer (Eddy and Shapiro, 1976). As early as 3 minutes after fertilization, these regularly arrayed microvilli have an elongated appearance and may become interconnected by lateral extensions (Eddy and Shapiro, 1976). Microvilli eventually become grouped and raised off the egg surface on mounds of cytoplasm (Chandler and Heuser, 1981). These cytoplasmic pedestals remain interconnected by lateral lamelli (Eddy and Shapiro, 1976; Chandler and Heuser, 1981).

In another species, S. drobachiensis, microvilli appear to remain unchanged in length and number for 60 minutes following the initial fertilization induced burst of elongation. Microvilli in this species show an average length of 0.5 μM, with less than 1.0% exceeding 1.0 μM in length. However, 30 to 90 minutes after fertilization, these microvilli show a second burst of elongation, attaining lengths up to 10 μM, with an average length of 1.8 μM (Schroeder, 1978).

The mechanism of elongation is postulated to operate via the controlled assembly of actin into microfilaments (Burgess, 1977; Spudich and Spudich, 1979; Begg and Rebhun, 1979; Burgess and Schroeder, 1977; Chandler and Heuser, 1981). As early as 1968,

Harris reported the presence of fibrils extending from microvilli into the subcortical cytoplasm. More recently, Kidd et al.(1976) demonstrated the presence of microfilaments associated with the plasmalemma of fertilized eggs in L. pictus, and Burgess and Schroeder (1977) have shown that egg microvilli contain polarized bundles of actin filaments capable of being decorated by heavy meromyosin. These microfilament bundles are anchored in the subcortical cytoplasm (Burgess and Schroeder, 1977; Chandler and Heuser, 1981).

Actin in the unfertilized egg appears to be present in a nonfilamentous form (Spudich and Spudich, 1979; Begg and Rebhun, 1979). This actin can be induced to polymerize by isolating egg cortices in buffers at a pH strength greater than 7.3 (Begg and Rebhun, 1979), and the associated microvilli of these isolated cortices are elongated with respect to microvilli of cortices isolated at lower pH (6.5 - 6.7). This suggests that actin polymerization resulting in elongated microvilli is under pH control. Nonfilamentous actin in cortices isolated in low pH (6.5), when transferred to pH 7.5, undergoes polymerization into microfilaments (Begg and Rebhun, 1979).

If pH is indeed the factor controlling actin polymerization and subsequent microvillar elongation,

agents such as NH_4Cl, which induce egg activation by elevation of internal egg pH, should also induce the elongation of microvilli. NH_4Cl is known to activate the late phase series of fertilization events while allowing the egg to bypass the early phase changes such as cortical granule exocytosis, and fertilization membrane elevation (see Chapter 2 for review of NH_4Cl induced egg activation and mechanism of action). It is not clear, however, if the egg is also bypassing surface alterations such as microvillar elongation. Mazia et al. (1975) has reported microvillar elongation in eggs of L. pictus and S. purpuratus activated with NH_4OH at pH 9.0 - 9.2, while Kidd et al. (1976) report plasmalemmal associated microfilaments in activated eggs. However, Carron and Longo (1980) were unable to demonstrate either microfilaments or elongated microvilli in eggs from Arbacia punctulata activated with NH_4Cl at pH 8.0.

In this paper, I demonstrate the presence of microfilaments and elongated microvilli in eggs from four species of sea urchins when activated with 10 mM NH_4Cl at pH 8.0. I have also correlated the presence of microvillar elongation with the presence or absence of the activating agent and with internal egg pH measured on egg homogenates.

MATERIALS AND METHODS

Lytechinus pictus, Strongylocentrotus purpuratus, Arbacia punctulata and Lytechinus variegatus were collected and maintained as described in Chapter 2.

Eggs, collected and washed as previously described (Chapter 2), were treated with dithiothreitol (Epel et al., 1970) to alter the vitelline layer and prevent fertilization membrane elevation. Eddy and Shapiro (1976) have shown that treatment with this agent, while causing morphological changes in microvilli, does not interfere with other surface events, including elongation of microvilli.

a. Egg Activation:

Demembranated eggs were activated in 10 mM NH_4Cl at pH 8.0. Eggs from a single female were divided into specific treatment groups as represented by the experiment outlined below for S. purpuratus:

Group 1 - Eggs were held in MBL-SW minus NH_4Cl for 290 minutes

Group 2 - Eggs were held in 10.0 mM NH_4Cl for 290 minutes

Group 3 - Eggs were treated with 10.0 mM NH_4Cl for 90 minutes, then washed into MBL-SW. NH_4Cl was readded at 225 minutes. Eggs were treated for a combined treatment time of 290 minutes.

Group 4 - Normal, fertilized eggs.

b. Internal pH:

Eggs from several females were checked for fertilizability and combined into a single batch to give a final egg concentration of 2.0 - 5.0%, depending on the species used. Internal pH was measured using egg homogenates according to the method of Johnson and Epel, 1975). pH was monitored on a Corning Model 12 pH meter.

c. Sample Fixation:

Samples for SEM were fixed in 2.5% (final) Glutaraldehyde-sea water (GTA-SW) for 24 hours, washed, and dehydrated in acidified 2,2 dimethoxypropane by the method of Muller and Jacks (1975). Dehydrated samples were critical point dried in acetone with CO_2, mounted onto cambridge SEM stubs with double stick tape, and sputter coated with 200Å of gold-palladium. Coated samples were viewed with an Hitachi S-500 Scanning Electron Microscope.

Samples for TEM were fixed on ice in 2.5% GTA in .45M sucrose-.1M PO_4 buffer as described by

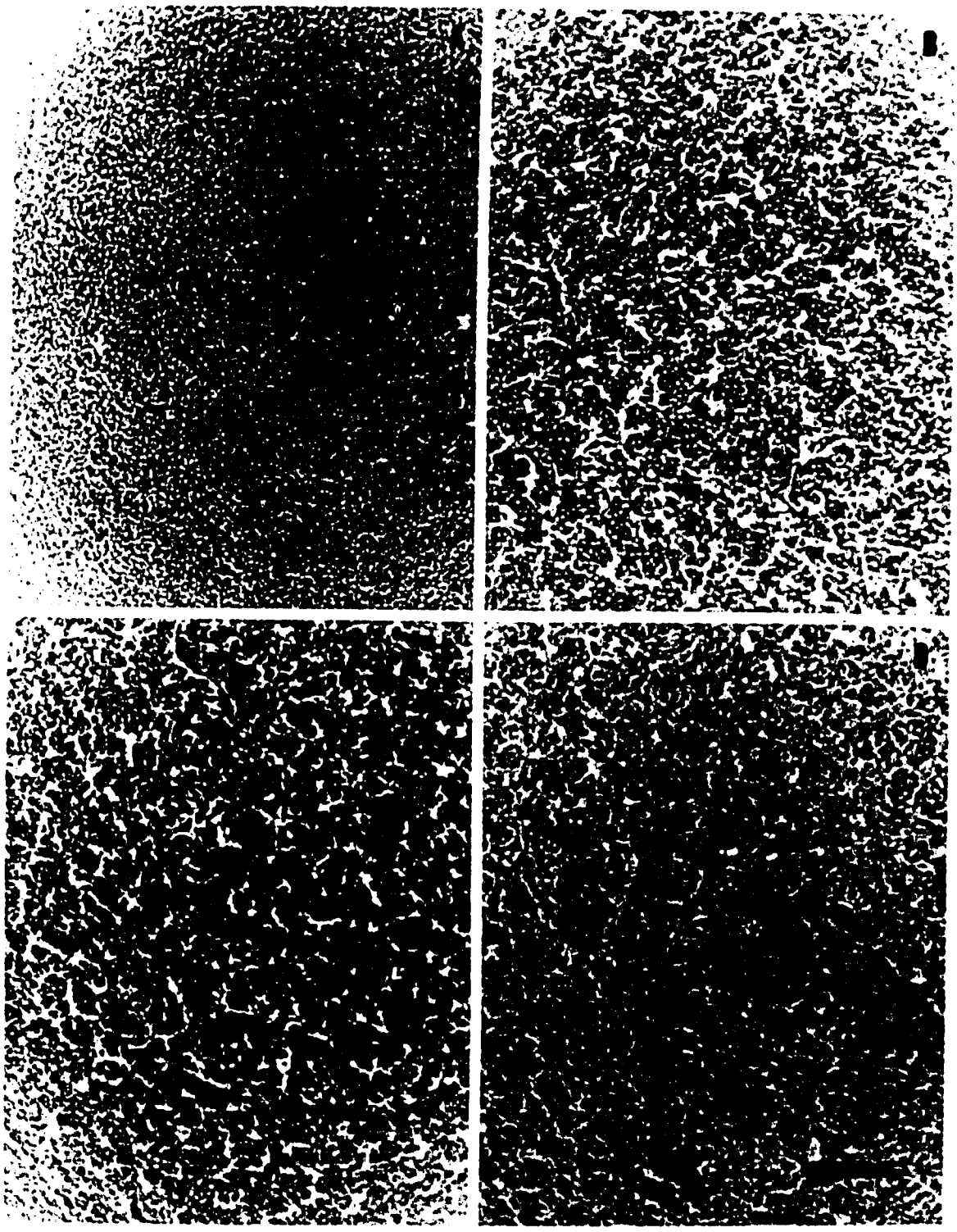
Gould-Somero and Holland (1975) for 1-3 hours with one fixative change. Fixed eggs were washed with three changes of sucrose-PO_4 buffer, and post-fixed in 1.0% osmium tetraoxide in 0.1 M PO_4 buffer for one hour at 4°C. Samples were washed with distilled H_2O and stained with .05% uranyl acetate. Eggs were dehydrated in a graded series of ethanol concentrations ranging from 30% to 100% (absolute), followed with several washes in 100% acetone. Dehydrated samples were impregnated with Spurr's resin, desiccated under vacuum, and transferred to fresh Spurr's resin in beem capsules. Polymerization was at 60° for 24 hours. Thin sections were post-stained with lead citrate and viewed with a JOEL 100CX Transmission Electron Microscope.

RESULTS

a. Surface Alterations in Activated Eggs:

Activation of eggs with 10 mM NH_4Cl (pH 8) does not result in immediate microvillar elongation. However, in all species studied, microvillar elongation was apparent by 60 minutes post-treatment. Results of an experiment using L. variegatus are shown in Figure 4-1(A-D). These results are representative of all species studied. In this experiment, eggs from a single female were divided into three groups. One group was held continuously in 10 mM NH_4Cl. The second group was treated with NH_4Cl for 70 minutes, and then washed into MBL-SW to remove the NH_4Cl. A control group was held untreated in MBL-SW for the duration of the experiment. Figure 4-1A shows a representative untreated egg from L. variegatus. The surface is covered by a regular array of evenly spaced microvilli. Figure 4-1B,C and D are representative of eggs held continuously in NH_4Cl for 60, 75, and 90 minutes, respectively. By 60 minutes, microvilli have begun to elongate, and by 70 minutes there is extensive elongation. The elongated microvilli are irregularly

Figure 4-1. Scanning electron micrograph of eggs from L. variegatus. A, untreated egg. Microvilli are short, regular and evenly spaced; B, egg after 60 minutes in 10 mM NH_4Cl. Microvilli are elongated and irregularly shaped; C, egg after 75 minutes in 10 mM NH_4Cl. Microvilli show prominent elongation; D, egg after 90 minutes in NH_4Cl. Microvilli are highly irregular and elongate. Size bar =5μ.



shaped, and do not appear to be interconnected by lateral lamelli. At 90 minutes post-activation (Figure 4-1D) the surface is completely irregular and covered with an extensive array of microvilli.

Eggs which are treated with NH_4Cl for 70 minutes, and then washed into MBL-SW no longer show a surface covered with elongated microvilli. Eggs sampled 15 minutes after washing (85 minutes of total treatment, figure 4-2) are characterized by a smooth surface, with few visible microvilli.

Treatment of eggs from S. purpuratus (as described in methods) results in similar microvillar elongation. Unfertilized, unactivated eggs show a regular array of evenly spaced microvilli (figure 4-3A). Transmission EM analysis of these eggs shows singly arranged, short microvilli. There is no evidence of any microfilament bundles traversing microvilli in these eggs (figure 4-3B, insert). Eggs treated continuously in NH_4Cl show elongation of microvilli beginning at approximately 60 minutes and continuing for the duration of the experiment. Microvilli in these eggs are irregularly shaped. Eggs sampled at 60, 150 and 210 minutes of continuous NH_4Cl treatment are shown in figures 4-4(A-C). TEM analysis of continuously treated eggs shows elongate microvilli containing

Figure 4-2. Scanning electron micrograph of *L. variegatus* egg treated for 70 min with 10 mM NH_4Cl, washed into MBL-SW, and sampled 15 minutes later. The surface is relatively smooth and microvilli are no longer prominent. Size bar = 5μ .

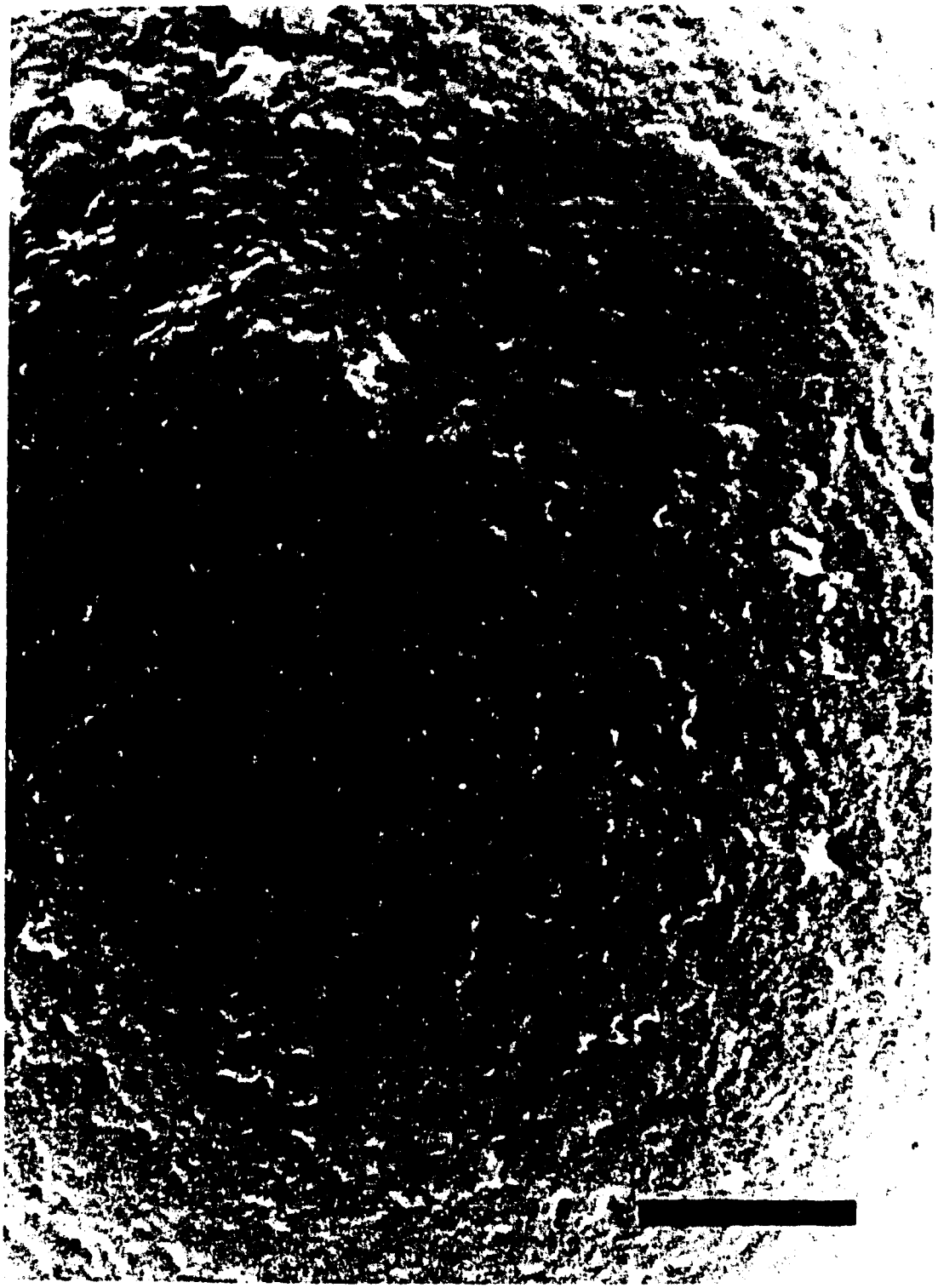


Figure 4-3. Control eggs from S. purpuratus.
A, SEM of a representative egg showing regular array of short, evenly spaced microvilli. Size bar = 5μ; B and C, Transmission electron micrograph of surface. Microvilli show relatively uniform diameter, even spacing, and lack of visible microfilaments.
B, Mag = 22,500 X; C, Mag = 40,000X

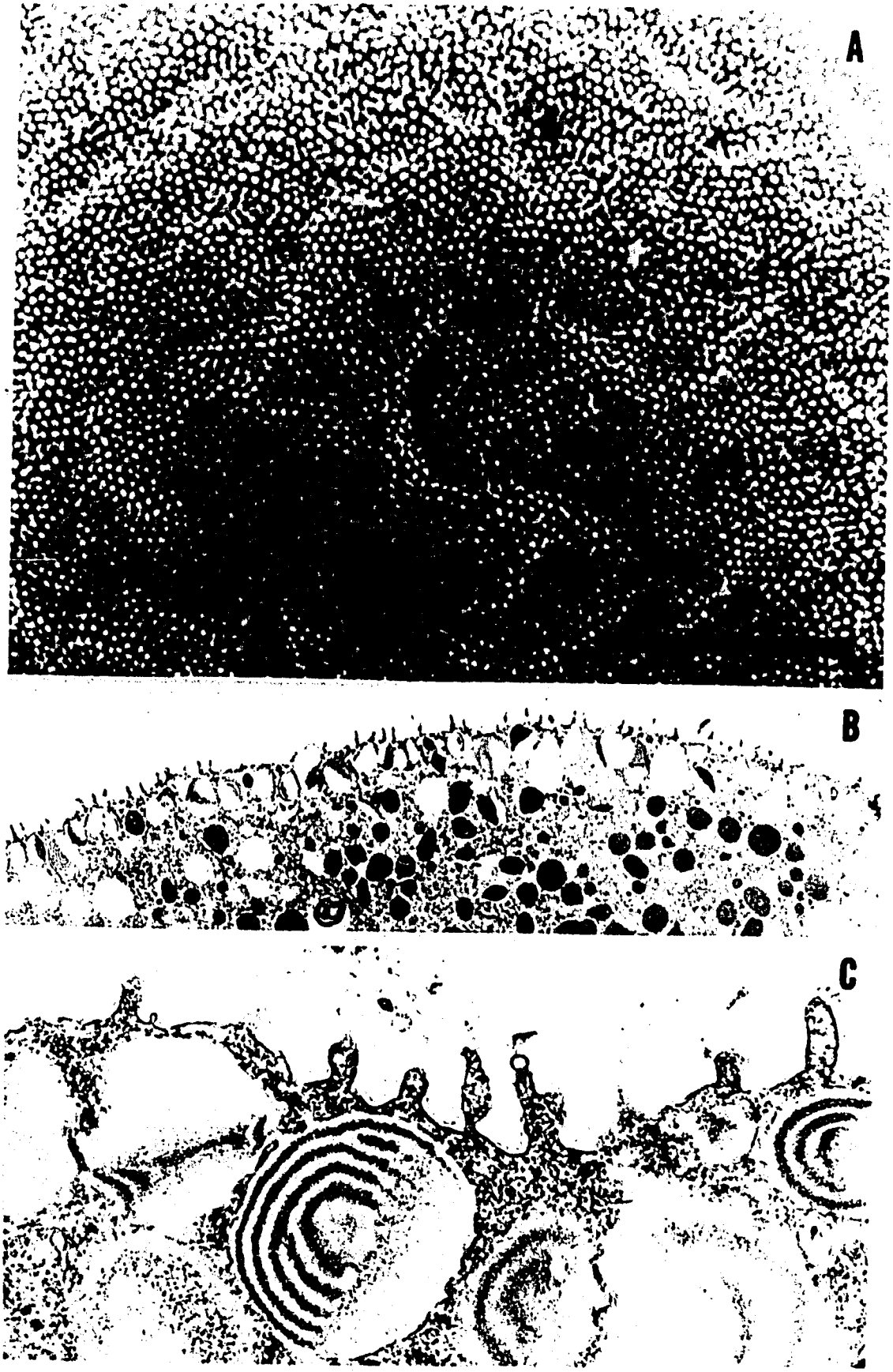
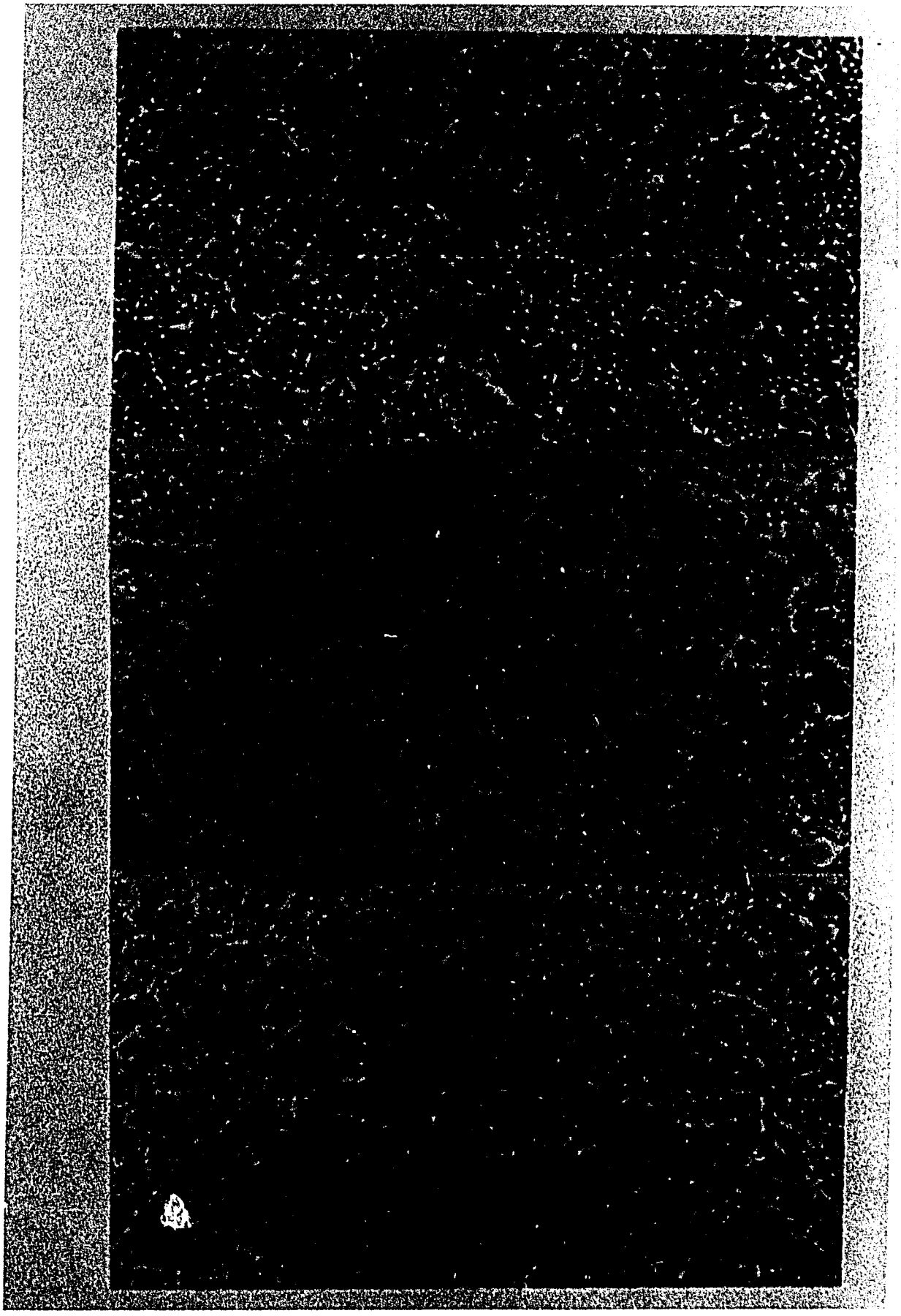

A
B
C

Figure 4-4. Egg from S. purpuratus held continuously in 10 mM NH_4Cl. Eggs were sampled after a, 60 min; b, 150 min; and c, 210 min. Microvilli are highly elongated and irregularly shaped.
Size bar = 5μ .



parallel bundles of microfilaments (figure 4-5, arrow). Eggs washed into MBL-SW at 90 minutes, and sampled at 195 minutes show considerably less microvillar elongation relative to eggs held continuously for the same period of time (figure 4-6, see figure 4-3C, 210 minutes of continuous treatment for comparative purposes). If NH_4Cl is readded to these eggs at 225 minutes, microvilli again show an extensive degree of elongation, and remain elongated even when total treatment time is as long as 6 hours (figure 4-7A,270 minutes; figure 4-7B, 6 hours).

When eggs washed at 90 minutes are sampled for transmission electron microscopy at 210 minutes, microvilli no longer have an elongated appearance. These microvilli are shortened, and appear to have increased in diameter (figure 4-8). There is no evidence of microfilaments within the microvilli of these eggs. When NH_4Cl is readded at 225 minutes, and eggs are sampled at 285 minutes for TEM, microvilli in these eggs once again assume an elongated state (figure 4-9). Microvilli are irregularly shaped and may even be branched or have a knobbed appearance. Microfilaments can be seen traversing the longitudinal axis of these microvilli (figure 4-9B, arrow).

Figure 4-5. Transmission electron micrograph of microvilli from the surface of an egg from S. purpuratus after continuous NH_4Cl treatment. Note the presence of longitudinally oriented microfilaments (arrow).
Mag = 78,000X.

Figure 4-6. Eggs from S. purpuratus treated with 10 mM NH_4Cl for 90 minutes, washed, and sampled at 195 minutes. Microvillar elongation is less prominent, but still visible.
Size bar = 5μ.

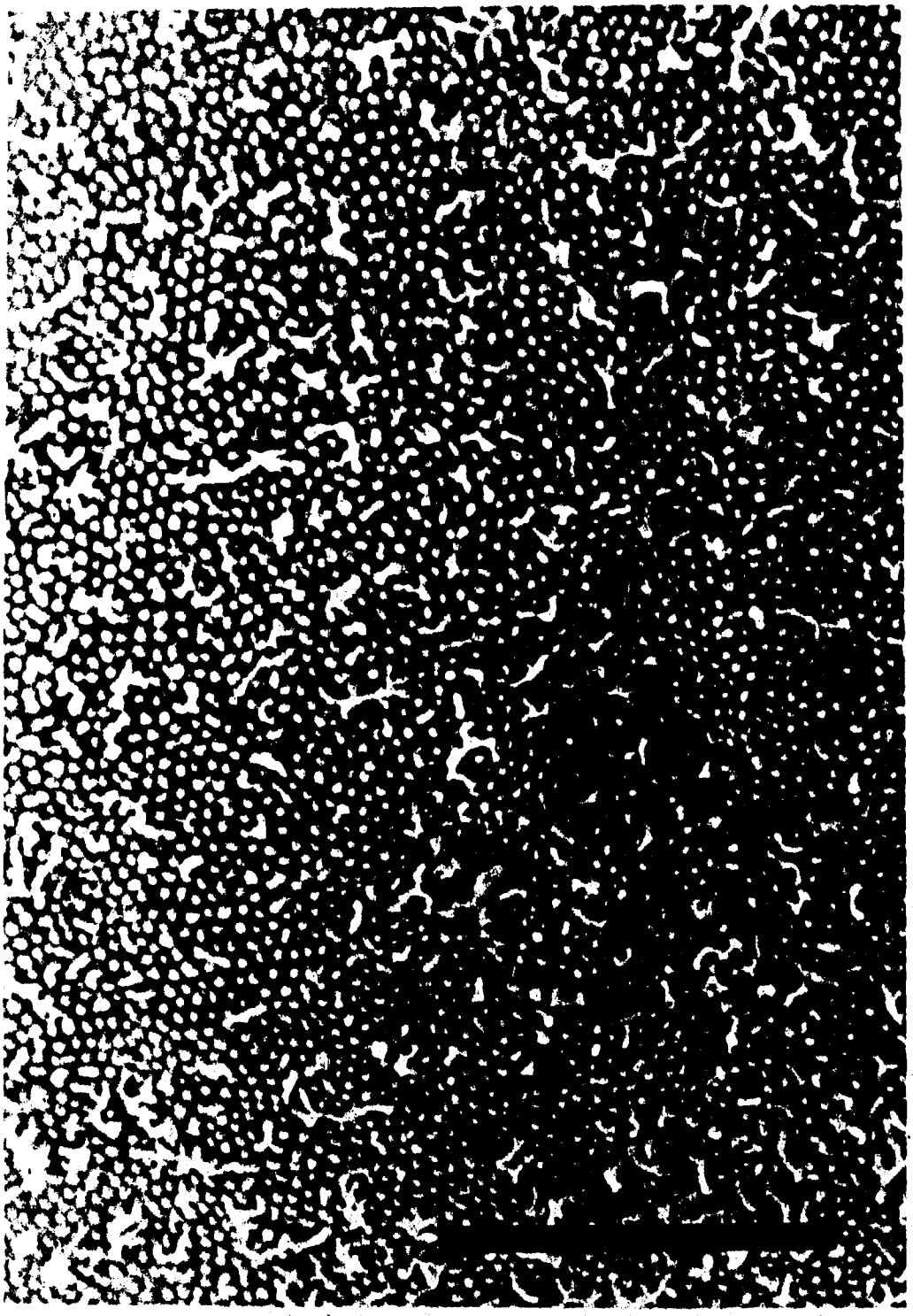


Figure 4-7. Eggs from S. purpuratus treated with 10 mM NH_4Cl for 90 minutes and washed into MBL-SW. NH_4Cl was readded to a final concentration of 10 mM 45 min later, and samples taken after 270 min (A) and 6 hours (B) of total combined treatment. Microvilli are irregularly shaped and elongated.
Size bar = 5μ.

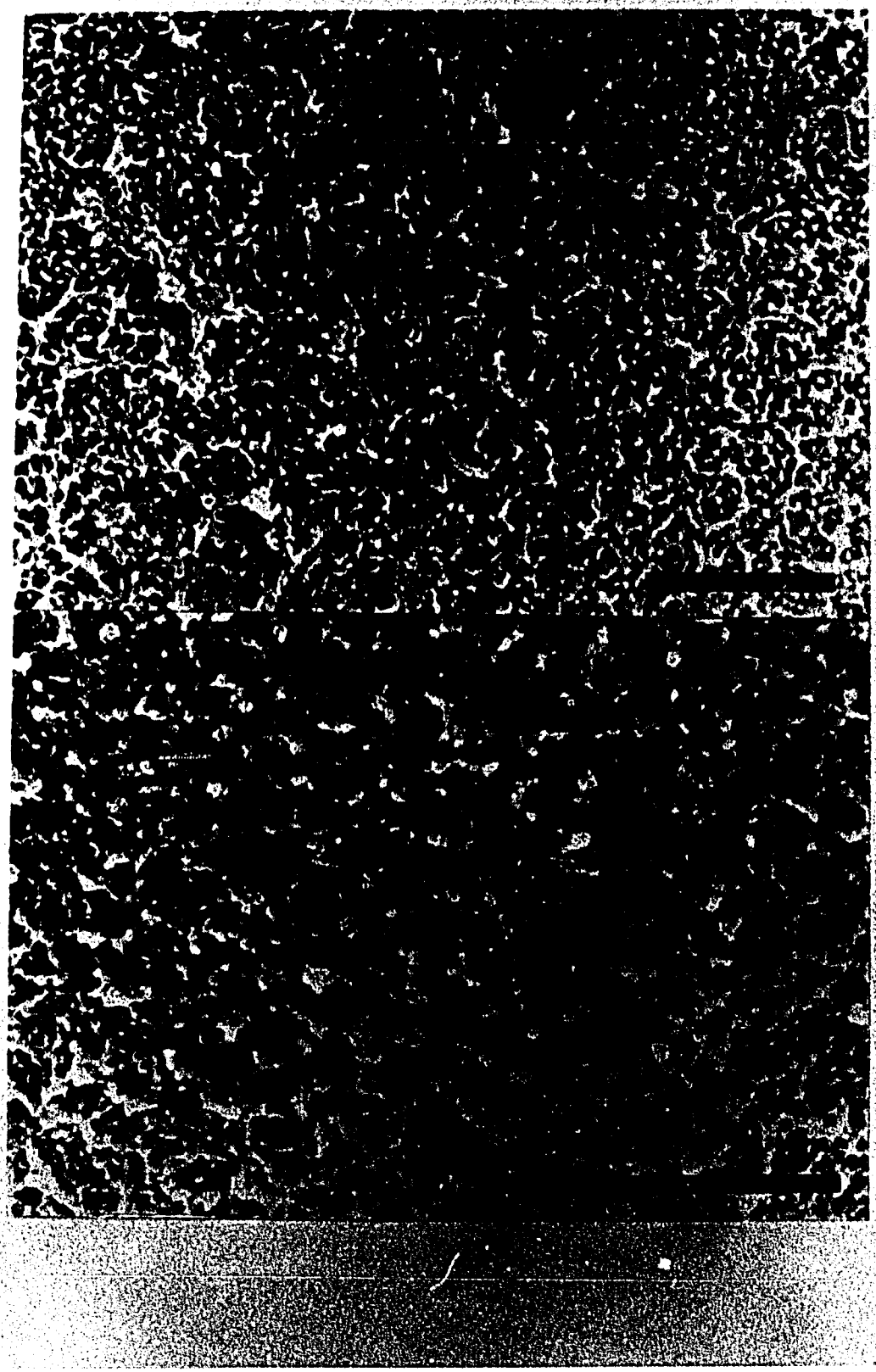
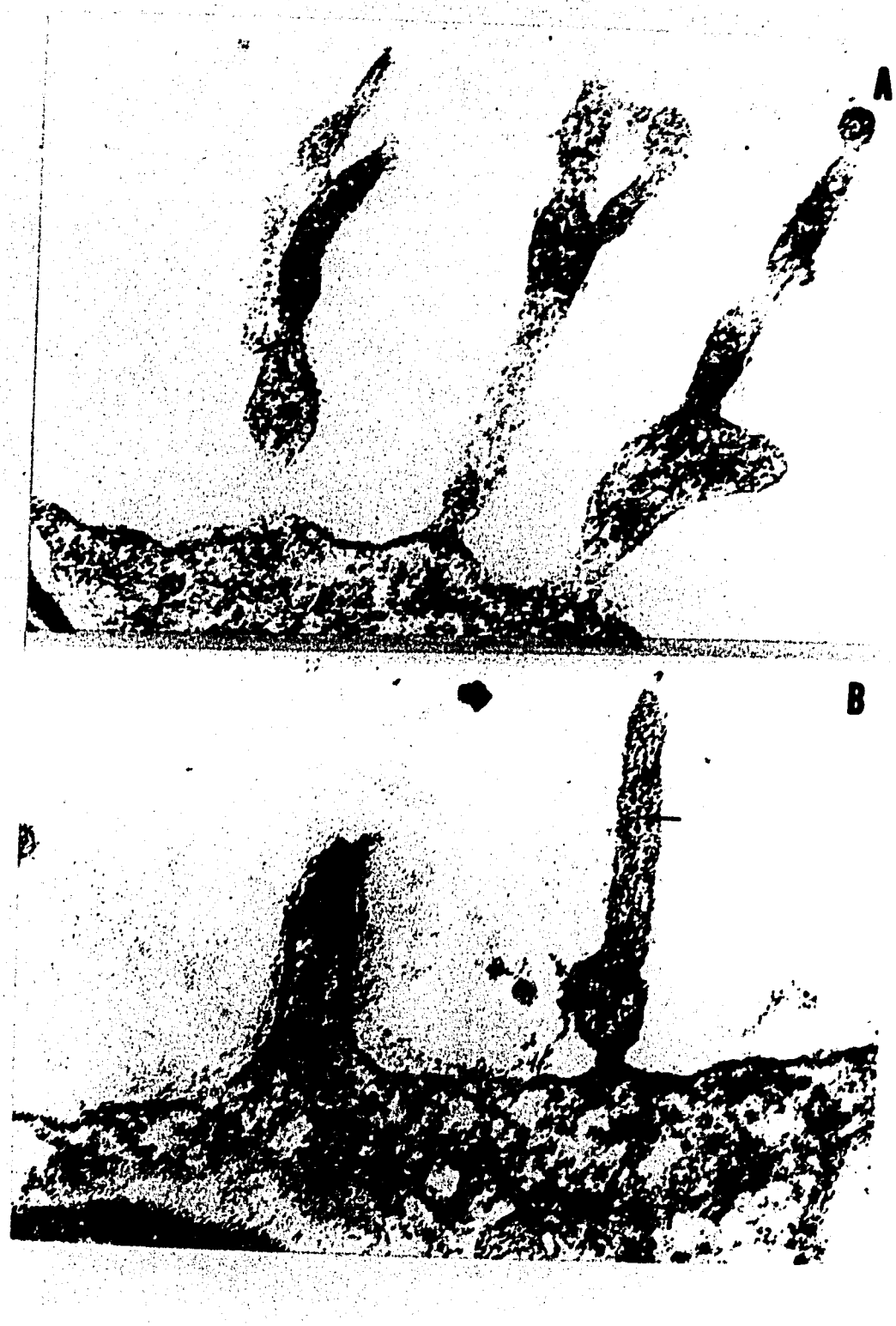


Figure 4-8. TEM of egg from S. purpuratus treated with 10 mM NH_4Cl for 90 min, washed, and sampled at 210 min. Microvilli are short, with increased diameters.
Mag. = 57,500X.

Figure 4-9. Egg from S. purpuratus treated for 90 min, washed for 45 min, and re-exposed to NH_4Cl. Eggs were sampled after a total treatment time of 275 min. A, Microvilli are irregular in shape, and may even appear to be knobbed and/or branched. Mag = 95,000 X. B, Microvilli are elongate, and contain a core of longitudinally oriented micro-filaments (arrow). Mag =114,000 X.


A
B

b. Internal pH in NH_4Cl Activated Eggs:

Results of internal pH measurments in S. purpuratus are shown in Table 4-1. The internal pH of unfertilized, unactivated eggs remained constant within .17 pH units. Fertilized eggs show an initial pH increase in egg homogenates over control values in the first 40 minutes, followed by a decrease in the next 40 minutes. Internal pH then fluctuated until the time of first cleavage. Internal pH in NH_4Cl activated eggs increased and remained in a more alkaline state relative to control eggs for the duration of the sampling period.

Table 4-2 shows similar results obtained for A. punctulata. In these experiments, one group of eggs was washed into MBL-SW at 90 minutes.

Table 4-1. Internal pH in S. purpuratus measured from egg homogenates. Eggs were treated with 10 mM NH_4Cl (pH 8.0), or fertilized. Values represent actual pH readings.

Time of Treatment	Control	Fertilized	NH_4Cl
0	6.25	-	-
10	-	6.39	-
20	-	6.35	6.85
30	-	6.46	-
40	6.32	6.45	6.85
50	-	6.42	-
60	-	6.39	-
70	-	6.37	-
80	6.32	6.26	6.78
90	-	6.40	-
100	-	6.37	-
110	-	6.31	-
120	6.42	6.37	6.38

Table 4-2. Internal pH in Arbacia punctulata measured from egg homogenates. Eggs are untreated, treated continuously, or treated for 90 minutes and washed into MBL-SW (WASH). Values represent actual pH readings.

Time of Treatment	Control	NH_4Cl	WASH
0	5.33	-	-
20	-	5.61	-
40	-	5.62	-
80	-	5.68	-
120	5.45	5.68	5.62
160	-	5.71	5.47
200	5.33	5.69	5.35

DISCUSSION

Sea urchin eggs undergo extensive surface alterations as a normal response to fertilization. Among these are cortical granule exocytosis, fertilization membrane elevation and hardening, and microvillar elongation (Guidice, 1973, review). Treatment of eggs with NH_4Cl does not result in cortical granule exocytosis or fertilization membrane formation (see Chapter 2 for review of NH_4Cl effects). However, I show here that microvillar elongation does occur after NH_4Cl initiated egg activation. This elongation appears to be a result of actin polymerization, as evidenced by the presence of microfilaments. Microvillar elongation is also dependent on the presence of the NH_4Cl. If the activating agent is removed, microvilli shorten, increase in diameter, and lose their microfilament core.

At present, the inter-relationships between cyclical surface changes, and cytoplasmic and nuclear events occurring throughout the cleavage cycle are unknown. There is some evidence that these events may occur independently (Kojima, 1980). For example,

eunucleate egg fragments, when activated with 1 M urea, are capable of undergoing cyclical surface alterations. These changes are not affected by addition of colcemid, which does cause suppression of cytoplasmic changes occurring as monitored by the appearance of sperm asters. In contrast, cytochalasin B does prevent surface alterations from occurring without interfering with cytoplasmic events. Both of these drugs prevent the cell from completing mitosis. It appears from these experiments that cyclical changes in the surface and cytoplasm are independent of nuclear control, and can also occur independently of each other, but that normal cleavage requires the interdependent occurrence of both cortical and cytoplasmic events (Kojima, 1980).

Alterations in surface ultrastructure, including changes in microvillar density and length, have been correlated with both cell division cycles and with cell shape changes in a variety of cellular systems. In sea urchin eggs, the total numbers of microvilli per square unit of surface does not change. However, two periods of microvillar elongation occur as a normal response to fertilization (Schroeder, 1978) in these types of cells. In other cell types which are characteristically flattened in shape as they enter mitotic stages, surface alterations are complex and difficult

to correlate directly with division cycles versus cell shape changes. In CHO cells (Porter et al., 1973) grown in tissue culture, cells in early G_1 are rounded with numerous surface microvilli and other cellular projections (blebs). As the cell traverses G_1, and the cell begins to lose its spherical shape, microvilli and blebs are present but not prominent. During S, when the cell is highly flattened, there are fewer microvilli; the flatter the cell, the fewer the microvilli. As the cell leaves S and enters G_2, the cell begins once again to thicken and microvillar density increases. Similar results have been seen in most cell types studied, including Hela (Pawelets, 1974a; 1974b), BHK 21 (Erickson and Trinkaus, 1976), and BHK 21/C13 (Follett and Goldman, 1970).

P8154 mastocytoma cells grown in suspension culture retain a spherical shape throughout the cell cycle. These cell types show surface changes similar to those discussed for static cultures (Knutton et al., 1975). P8154 cells in G_1 are relatively smooth, with surface microvilli exhibiting uniform diameters. As the cells traverse S and G_2, microvilli become irregular, highly branched, and large surface blebs form.

The function of microvillar elongation and/or increase in density is unknown. It has been suggested

that microvilli may serve as one mechanism of membrane conservation or storage during cell rounding or cell division (Wolpert, 1963; Erickson and Trinkaus, 1976; Knutton et al., 1975). Erikson and Trinkaus also suggest that, in addition to providing a source of reserve membrane material during cell flattening, microvilli may serve as the structural basis for membrane redistribution, with the polarized microfilament core serving as the generating force in the shifting of membrane material from one surface site to another.

Formation of microfilament bundles in NH_4Cl activated eggs was not surprising. NH_4Cl is postulated to initiate egg activation through its effects on egg internal pH (Winkler and Grainger, 1978). Increased intracellular pH is known to initiate polymerization of actin involved in the sea urchin sperm acrosome reaction (Tilney et al., 1977), and isolation of cortices from sea urchin eggs at pH's above 7.3 induces filament formation and microvillar elongation (Begg and Rebhun, 1979). In addition, cortices isolated at pH 6.5 and then transferred to pH 7.5 are induced to polymerize actin, although filament polarity in these preparations is lost. In contrast, cortices isolated from fertilized eggs showing

filamentous actin are not affected by isolation in low pH. Actin polymerization under these conditions is not reversible. We have found the opposite to be true in NH_4Cl treated eggs. If NH_4Cl is removed, microvilli decrease in length, but increase in diameter. There is no evidence of filament bundles in samples examined under TEM, although it is possible that filament polarity has been lost, making identification of microfilaments difficult. NH_4Cl does not result in immediate return to unfertilized levels (Table 4-2), at least in Arbacia. In fact, 30 minutes after removal, internal pH remained relatively unchanged. However, these pH measurements were recorded from egg homogenates, and do not reflect egg compartmental differences in pH which may occur within whole eggs.

In summary, it appears from these experiments that NH_4Cl is capable of inducing microvillar elongation and actin polymerization in sea urchin eggs. These effects are probably mediated through increased intracellular pH brought on by NH_4Cl addition.

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VITA

Barbara Ellen Belisle (nee Wolfanger) was born in North Hornell, New York. She attended Hornell Sr. High School and graduated with a New York State Regents Diploma in 1969. She received a Bachelor of Arts with Departmental Honors in Biology from Keuka College, N.Y. in 1973. She was employed by the W. Alton Jones Cell Science Center as a junior laboratory technician from 1973-1974, and by Dr. Simon H. Chang, Department of Biochemistry, LSU from 1974-1975 as a research associate. She entered Louisiana State University as a full time graduate student in the Department of Zoology and Physiology in August, 1975, and received a Masters of Science Degree in Physiology in August, 1977. She is presently a candidate for the Doctor of Philosophy Degree in Physiology under the direction of Dr. E. W. Byrd.

EXAMINATION AND THESIS REPORT

Candidate: Barbara Ellen Wolfanger Belisle

Major Field: Physiology

Title of Thesis: Mitotic Triggers in the Cell Cycle: Phase Dependency of Cleavage Advance on the Chromosome Cycle in Parthenogenetically Activated Sea Urchin Eggs.

Approved:

W. Byrd
Major Professor and Chairman

James D. Graynham
Dean of the Graduate School

EXAMINING COMMITTEE:

J P Woodard

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Date of Examination:

June 25, 1981